REMARKS

This Amendment, filed in reply to the Final Office Action dated July 9, 2010, is believed to be fully responsive to each point of objection and rejection raised therein. Accordingly, favorable reconsideration on the merits is respectfully requested.

Claims 1-15 and 17-28 are all the claims pending in the application and all are rejected.

Claims 1, 2 and 26 are amended to require "adapting the connective tissue matrix to increasing flow rates," an "adapted, solid connective tissue matrix structure," "at least approximately 8 days post colonization" and "an autologous tissue-engineered heart valve that can withstand flow rates of more than 2,000 ml/min."

These claim amendments are being made herewith solely to improve clarity and conciseness. Support for these amendments can be found throughout the specification as filed.

Specifically, support for the adaptation of the connective tissue matrix to increasing flow rates can be found at the following locations of Applicant's published patent application US 2006/0246584:

In paragraph [0025], which states:

> ". . . . According to the invention, the preformed structure analogous to a heart valve can now be introduced, in a further method step for maturing the tissue and optimizing the haemodynamic function, into a pulsatile flow chamber in which it can be exposed to increasing flow rates. **By continuous or discontinuous increasing of the flow rate, it is adapted here to the flow conditions in the human body**. For further stabilization, the structure analogous to a heart valve is fixed to a biocompatible frame construction of non-degradable or poorly degradable material, which is optionally introduced again into the pulsatile flow chamber. In the case where **adaptation to the flow conditions in the human heart** is carried out in the flow chamber after fixing to the frame construction, the first incubation in the pulsatile flow chamber can be omitted. **Vital heart valve**

> **prostheses which withstand the flow conditions in the human body are obtained by these methods**." (Emphasis added)

In paragraph [0030], which states:

> "[0030] **adaptation** in a pulsatile flow chamber." (Emphasis added)

In paragraph [0036] which states:

> "[0036] **adaptation** of the "stented" heart valve." (Emphasis added)

In paragraph [0039], which states:

> "[0039] **adaptation**" (Emphasis added)

In paragraph [0061] which states:

> "[0061] According to the invention, . . . **formation of a connective tissue matrix which is resistant to flow can be achieved by slow adaptation of the flow rates**."
> (Emphasis added)

Support for the term a "solid connective tissue matrix structure" can be found in paragraph [0024] of Applicant's published patent application US 2006/0246584, which states:

> "[0024] After formation of **a solid connective tissue matrix structure**, the degradable support of which does not yet have to be dissolved, this is optionally colonized with endothelial cells. After the colonization has taken place, the connective tissue matrix is applied to a non-degradable or poorly degradable frame construction." (Emphasis added)

Support for the term "approximately" can be found in paragraph [0023] of Applicant's published patent application US 2006/0246584, which states:

> ". . . It is preferable for the degradation to start after **approx.** 8 days; as a rule, it should be concluded in less than 3 months, preferably already after 4 to 6 weeks." (Emphasis added)

Support for the phrase "a tissue-engineered heart valve that can withstand flow rates of more than 2,000 ml/min, corresponding to the flow conditions prevailing in an adult human heart" can be found in paragraph [0068] of Applicant's published patent application US 2006/0246584, which states:

> "[0068] It was possible to demonstrate that the **heart valves according to the invention withstand flow rates of more than 2,000 ml/min, corresponding to the flow conditions prevailing in an adult human heart**." (Emphasis added)

Applicant's also assert a person of ordinary skill in the art would understand from Applicant's disclosure that after complete degradation of the "biodegradable support" and the "slowly degradable frame, " the claimed invention provides an autologous tissue engineered heart valve that no longer has any external support because the biodegradable support and the slowly degradable frame have degraded away.

No new matter is added by way of these claim amendments. Entry and consideration of this amendment are respectfully requested.

Correction of the English Translation of the corresponding PCT application

The PCT application, PCT/EP2002/009906, that is incorporated by reference into the present application (see Preliminary Amendment, dated February 1, 2005, and citations in the next section) states on page 6 between lines 21 and 23:

> "Bei dem Tragermaterial handelt es sich bevorzugt um eine aus Polymerfasern aufgebaute Struktur, um eine porose Polymerstruktur oder ein azellulares biologisches Gewebe."

The English translation of this German phrase can be found on page 6 of the specification as filed, between lines 10 and 17, which states:

> "The support material is preferably a structure built up from polymer fibres around a porous polymer structure or an acellular tissue."

Applicants assert the German word "um" was improperly translated to the term "around." The German term "um" can either mean "around" or, together with the use of the German term "handelt es sich... um" (see lines 1-2 of paragraph 2 of page 6 of the International Application WO 2004/018008 quoted above), it is a preposition used in connection with a verb. Therefore, the passage is supposed to specify a list of three different possible support materials, and not one material arranged "around" another.

Accordingly, Applicants request the paragraph on page 6 be amended to have the correct English translation as follows:

> "The support material is preferably a structure built up from polymer fibres, ~~around~~ or a porous polymer structure, or an acellular tissue".

35 U.S.C. § 112 rejection - Written description

Claims 1-28 are rejected under 35 U.S.C. § 112, first paragraph, as allegedly failing to comply with the written description requirement. The Examiner contends the claim amendment "slowly degradable" cannot be found in the specification as filed and thus it constitutes new matter.

The Applicant respectfully traverses the rejection for the reasons outlined below. Applicant asserts original claim 26 of the present application and the PCT application, PCT/EP2002/009906, requires "a slowly degradable frame." Specifically, original claim 26 of the present application, filed on February 1, 2005, states:

> "26. Autologous heart valve having a connective tissue inner structure surrounded by an endothelial cell layer, characterized in that it is fixed to a non-degradable or slowly degradable frame construction (stent)."

In addition, the Preliminary Amendment filed concurrently with the present Application on February 1, 2005, amended the specification to require the insertion of the following reference to related applications at page 1, line 3 of the specification:

> "This application is a national stage application, filed under 35 U.S.C. 371, of PCT/EP2002/009906, filed on September 4, 2002, which claims priority to German Application No. 10235237.2, filed on August 1, 2002. Each of these applications is herein incorporated by reference in its entirety."

Thus, original claim 26 of the PCT application, PCT/EP2002/009906, that also requires "a slowly degradable frame," is incorporated by reference into the present application.

Applicants conclude a "slowly degradable frame" is not new matter because this language can be found in the specification as filed and the corresponding PCT application, PCT/EP2002/009906, which is incorporated by reference into the present application.

Applicants therefore request that the rejection of claims 1-28 under 35 U.S.C.§ 112, first paragraph, be withdrawn.

35 U.S.C. § 103 rejection - Obviousness

Claims 1-15 and 18-28 are rejected under 35 U.S.C. 103(a) as being obvious in view of Hoerstrup DE 19919625 (herein after "Hoerstrup") further in view of Arru U.S. Patent No. 4,758,151 (hereinafter "Arru").

The Examiner states Hoerstrup discloses providing a biodegradable support, colonizing the support with homologous fibroblast and/or myofibroblasts cells to form a connective tissue matrix; optionally colonizing the connective tissue matrix with endothelial cells; fixing of the

matrix to a non-degradable or poorly degradable frame construction. Hoerstrup discloses the connective tissue matrix is introduced into a pulsatile flow chamber in which it can be exposed to increasing flow rates, and the flow rate is increased continuously or discontinuously. Further, the Examiner states Hoerstrup teaches a material that degrades at least 8 days post colonization and is completely degraded within four to six weeks.

According to the Examiner, Applicant's disclosure states that both the "non-degradable or poorly degradable frame construction" and "biodegradable support" can be constructed of PHA. Because both can be made of the same material, the Examiner alleges the use of the terms "biodegradable" and "poorly degradable" becomes non-limiting.

The Examiner admits Hoerstrup fails to teach a poorly degradable frame construction where the frame does not degrade prior to a year after colonization. The Examiner asserts Arru teaches the allegedly well-known practice of using pulsatile flow chambers to construct biodegradable supports and attach them to stents or other support structures, that are solid, such as a suture ring.

The Examiner then alleges it would be obvious to one of ordinary skill in the art at the time of invention to modify Hoerstrup in view of Arru, in order to use a known stent support structure material with an easily degradable connective tissue matrix, as is disclosed in Hoerstrup and Arru. According to the Examiner, if the claimed and prior art products are identical in composition, a prima facie case of either anticipation or obviousness has been established.

The Applicant respectfully traverses the rejection for the following reasons.

*1) **DE 19919625 publication is not prior art under 35 U.S.C 102(a) because it describes the Applicant's own work***

Applicants refer the Examiner to MPEP 715.01(c), section 1, which states:

> I. CO-AUTHORSHIP
> Where the applicant is one of the co-authors of a publication cited against his or her application, he or she may overcome the rejection by filing an affidavit or declaration under 37 CFR 1.131. Alternatively, the applicant may overcome the rejection by filing a specific affidavit or declaration under 37 CFR 1.132 establishing that the article is describing applicant's own work. An affidavit or declaration by applicant alone indicating that applicant is the sole inventor and that the others were merely working under his or her direction is sufficient to remove the publication as a reference under 35 U.S.C. 102(a). In re Katz, 687 F.2d 450, 215 USPQ 14 (CCPA 1982).

Dr. Hoerstrup, one of the Inventors of the present Application, submits herewith an Affidavit under 37 CFR 1.132 in which he declares he is one of the authors and one of the inventors of the DE 19919625 patent publication cited by the Examiner in the 35 U.S.C § 103 rejection of the Final Office Action dated July 9, 2010. Dr. Hoerstrup also clarifies his statements made in the German patent application, DE 19919625 and then explains the differences between his present invention and the DE 19919625 reference.

Applicants assert the DE 19919625 Hoerstrup reference is a publication of the Applicant's own work that does not describe work "by another" as required by 35 U.S.C § 102(a). Applicants therefore submit the DE 19919625 Hoerstrup reference is not prior art with respect to Applicant's invention and respectfully request that the rejection of the claimed invention under 35 U.S.C § 103 in view of Hoerstrup and Arru be withdrawn.

2) The combination of the Hoerstrup and Arru references fails to teach or suggest every limitation of Applicant's claimed invention

To support an obviousness rejection, MPEP §2143.03 requires "all words of a claim to be considered" and MPEP § 2141.02 requires consideration of the "[claimed] invention and prior art as a whole." Further, the Board of Patent Appeals and Interferences confirmed that a proper, post - KSR obviousness determination still requires the Office make "a searching comparison of the claimed invention – **including all its limitations** – with the teaching of the prior art." In re Wada and Murphy, Appeal 2007 - 3733, citing In re Ochiai, 71 F.3d 1565, 1572 (Fed. Cir. 1995) and CFMT v. Yieldup Intern. Corp., 349 F.3d 1333, 1342 (Fed. Cir. 2003).

In sum, it remains well-settled law that an obviousness rejection requires at least a suggestion of all of the claim elements.

a) Arru fails to teach Applicant's "slowly degradable frame"

At the top of page 6, the Examiner admits that Hoerstrup fails to teach a "slowly degradable frame construction" but that Arru teaches the well-known practice of using pulsatile flow chambers to construct biodegradable supports and attach them to stents.

Applicants disagree and assert the Arru reference fails to disclose a "slowly degradable" frame construction that does not degrade prior to a year after colonization as required by Applicant's claimed invention. Hence, Applicants affirm that the Hoerstrup and Arru references fail to teach or suggest each and everyone of the limitations of Applicant's claimed invention.

b) The "adapted, solid connective tissue matrix structure" is distinct from the "slowly degradable frame construction"

The Examiner alleges the addition of a "support" structure to Hoerstrup's design is obvious, since it can be made of the same material, and it has been held that the selection of a known material based on its suitability for its intended use supports a *prima facie* case for obviousness. Specifically, the Examiner states both the "non-degradable or poorly degradable frame construction" and "biodegradable support" can be constructed of PHA. Since both can be made of the same material, the use of the terms "biodegradable" and "poorly degradable" becomes allegedly non-limiting.

Applicants respectfully disagree.

i) The composition of the connective tissue structure is different from the frame construction

The initial matrix, optionally made of PHA, is but one component of the final "solid connective tissue matrix structure." Claims 1 and 2 require seeding of the porous biodegradable support with fibroblasts or myofibroblasts that infiltrate into the matrix. A person of ordinary skill in the art would know that cells form a tissue by secreting a complex extracellular matrix. The slow conditioning of the nascent tissue to a pulsatile flow in a bioreactor results in (1) the organization of the heart valve tissue, and (2) the concomitant deposition of an extracellular matrix characteristic of human heart valves by the colonizing cells.

Specifically, the specification as filed states in paragraph [0067] of Applicant's published patent application US 2006/0246584:

> "[0067] The heart valve according to the invention comprises a **connective tissue inner structure** which contains, in addition to fibroblasts and myofibroblasts, substantial **constituents of a**

> **normal extracellular matrix**, namely collagen, elastin and glycosaminoglycans. The valves according to the invention thus have a content of **collagen (26-60%), elastin (2-15%) and glycosaminoglycans** corresponding to the native valve or the native valve leaflet. This **connective tissue inner structure built up on a biodegradable support** (scaffold) and colonized with endothelial cells is stabilized further by a biocompatible frame construction. The connective tissue structure is fixed to the biocompatible frame construction as described above." (Emphasis added)

Applicants therefore assert the "solid connective tissue matrix structure" is not just a matrix composed of PHA. On the contrary, the slow adaptation of the colonizing cells to increasing flow rates results in the secretion of a complex extracellular matrix and the formation of a heart valve tissue.

ii) The mechanical properties of the connective tissue structure are different from those of the frame construction

A person of ordinary skill at the time of Applicant's filing would know the interstitial cells and the deposited extracellular matrix of the connective tissue structure confer on the nascent tissue properties characteristic of heart valves that permit applicant's tissue engineered heart valves to withstand flow rates of more than 2,000 ml/min.

The properties of the different components of a heart valve are reviewed below (reproduced from Sacks et al. 2009[1,2] and Schoen, 1997[2,3]).

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[1] Bioengineering Challenges for Heart Valve Tissue Engineering Sacks MS, Schoen FJ, Mayer JE. Annu Rev Biomed Eng. 2009;11:289-313.

[2] In accordance with M.P.E.P. 609(c), the documents cited herein in support of Applicants' remarks are being submitted as evidence directed to an issue raised in the Official Action, and no fee pursuant to 37 C.F.R. 1.97 or 1.98, or citation on a FORM PTO/SB/08 or PTO-1449 is believed to be necessary.

[3] Schoen F. 1997. Aortic valve structure-function correlations: role of elastic fibers no longer a stretch of the imagination. J. Heart Valve Dis. 6:1–6

Table 1 Key cellular and extracellular matrix components of the aortic valve (modified from Reference 19).

Component	Location	Putative Function	Comments, key questions
Endothelial cells	Lining inflow and outflow valve surfaces	Provide thromboresistance, mediation of inflammation	Role in transducing shear and modulating VIC function, functional differences from vascular wall EC, differences in inflow side to outflow side functions/responses largely unknown
Interstitial cells	Deep to surface, throughout all layers	Synthesize and remodel matrix elements	Currently considered the major modulator of long-term valve durability and a key mediator of disease; regional heterogeneity; regulation of activation, and the functional role of contractile potential poorly understood
Elastin	Concentrated in ventricularis layer	Extend in diastole, recoil in systole	Potential mechanistic role in disease not defined
Glycosaminoglycans (GAGs)	Concentrated in spongiosa layer	Absorb shear of relative movements and cushion shock between ventricularis and fibrosa during cyclical valve motion	Potential mechanistic role in disease not defined
Collagen	Concentrated in fibrous layer	Provides strength and stiffness to maintain coaptation during diastole	Likely the most important structural element, crimp and orientation/alignment provide directional anisotropy of properties and accommodate cyclical cuspal shape changes

In contrast, paragraph [0066] of Applicant's published patent application US 2006/0246584 states:

> "Thus, in its preferred embodiment, the heart valve according to the invention comprises autologous tissue, i.e. tissue of the patient scheduled for the heart valve operation, and a biocompatible material which **stabilizes it further**, which is used as the frame construction." (Emphasis added)

Thus, the frame construction fills a role of stabilizing the heart valve prosthesis.

The properties of the "connective tissue inner structure built up on a biodegradable support" include elasticity of the structure whereas the frame / stent provides stability to the construct. Applicants conclude the connective tissue structure is not the same as the frame construction because their biophysical properties are different.

iii) The support and the frame degrade at different rates

In their response dated July 29, 2009, Applicants amended claims 1, 2 and 26 to require that the biodegradable material begins degrading at least approximately 8 days after colonizing

the device with cells and completes degradation by, at most, 3 months. The Applicants also amended claims 1, 2 and 26 to specify that the poorly degradable material does not degrade for longer than a year after colonizing the device with cells. Thus, despite the possibility that PHA could be used, at least in part, to construct either material, the amount of PHA used and the other components that PHA is combined with differentiate a biodegradable material from a slowly degradable material.

3) Arru teaches away from Applicant's invention

It is well-settled law that a prima facie case of obviousness may be rebutted by showing that the art teaches away from the claimed invention. See In re Geisler, 116 F.3d 1465, 1471, 43 USPQ2d 1362, 1366 (Fed. Cir. 1997). Further, relevant law holds that a reference teaches away when a person of ordinary skill in the art, upon reading it, would be discouraged from following the path set out in the reference, or would be led in a path divergent from the path taken by the inventor. See Monarch Knitting Mach. Corp v. Sulzer Morat Gmbh, 139 F.3d, 877, 45 USPQ2d 1977 (Fed. Cir. 1998); Para-Ordnance Mfg. v. SGS Importers Int'l Inc., 73 F.3d1085, 37 USPQ2d 1237 (Fed. Cir. 1995); and In re Gurley, 27 F.3d 551, 553 (Fed. Cir. 1994).

Arru contains information relating to the production of heart valve implants using tissue sheets from animals (col. 1, lines 23-25). The Arru patent neither teaches nor suggests a biodegradable frame. For example, in col. 5, lines 35-42 Arru teaches metallic or non-biodegradable materials:

> "The frame of the prosthesis, generally indicated, includes **a rigid or semi-rigid stent** having a set of three shaped projections (FIG. 3).
>
> The stent 3 and the projections are normally constituted by a single piece of biocompatible material such as, for example, **titanium, a chrome-cobalt alloy or one based on cobalt, or else the plastics**

materials known by the commercial names "Teflon" or "Delrin".

Arru guides the ordinarily skilled artisan to a divergent process and product, not the claimed invention. A key aspect of Applicant's invention is the conditioning of the heart valve tissue **in combination with** the biodegradable support / slowly degradable frame until stents or other support mechanisms are no longer required. The progressive weakening of the heart valve caused by the gradual degradation of the frame induces a concomitant adaptation of the heart valve itself to ever increasing stresses present within the heart. Eventually, the heart valve sustains the stresses in the heart without any support or stent. Applicant's slowly degradable frame therefore provides **transient** support during the adaptation of the tissue engineered heart valve to the hemodynamic stresses within the heart. In contrast, Arru's stent is intended to provide **continuous** support for tissue sheets devoid of any internal structure or strength.

Hence, a person of ordinary skill would not be motivated to combine Hoerstrup and Arru, because Arru's stent would not permit the progressive adaptation of the autologous tissue engineered heart valve to a point where the stent is no longer required for the proper function of the heart valve in the heart.

4) Success in the field of tissue engineered heart valves is highly unpredictable hence non-obvious

In the wake of the decision by the Supreme Court in KSR International Co. v. Teleflex Inc., the Office established Guidelines that should be followed in making an obviousness determination. The Guidelines indicate a rationale must be set forth as to why the claimed invention is obvious. The Guidelines indicate the following rationales are indicative of obviousness:

(a) combining prior art elements according to known methods to yield ***predictable*** results;
(b) simple substitution of one known element for another to obtain ***predictable*** results;
(c) use of a known technique to improve similar devices (methods, or products) in the same way;
(d) applying a known technique to a known device (method, or product) ready for improvement to yield ***predictable*** results;
(e) "Obvious to try"—choosing from a finite number of identified, ***predictable*** solutions, with a reasonable expectation of success;
(f) known work in one field of endeavor may prompt variations of it for use in either the same field or a different one based on design incentives or other market forces if the variations would have been ***predictable to one of ordinary skill in the art***;
(g) some teaching, suggestion, or motivation in the prior art that would have led one of ordinary skill to modify the prior art reference or to combine prior art reference teachings to arrive at the claimed invention. (Emphasis added)

Thus, according to the Guidelines, predictability to one of ordinary skill in the art is a key determinant in an obviousness analysis, particularly in an unpredictable art such as tissue engineering.

On page 7 of the Office Action, the Examiner cites the rationale for obviousness in which the combination of prior art elements according to known methods yields predictable results.

Applicants assert this rationale does not apply to Applicant's invention. On the contrary, the state of the art at the time of Applicant's invention clearly shows the construction of tissue engineered heart valves that can withstand the hemodynamic stresses found in the human aorta was far from obvious.

For example, Ivan Vesely's review article[4] entitled "Heart Valve Tissue Engineering" that was published three years after Applicant's earliest effective filing date, illustrates the failure

[4] Vesely I., Heart valve tissue engineering Circ Res. 2005 Oct 14;97(8):743-55. In accordance with M.P.E.P. 609(c), the documents cited herein in support of Applicants' remarks are being submitted as evidence directed to an issue raised in the Official Action, and no fee pursuant to 37 C.F.R. 1.97 or 1.98, or citation on a FORM PTO/SB/08 or PTO-1449 is believed to be necessary.

of others to engineer a durable heart valve as well as the unpredictability in the field of tissue engineering.

Specifically, Vesely states in the abstract:

> "Two main approaches have been attempted over the past 10 to 15 years: regeneration and repopulation. . . . . ***Regrettably, neither of the 2 approaches has fared well in animal experiments, and the only clinical experience with tissue-engineered valves resulted in a number of early failures and patient death.***" (Emphasis added)

The author proceeds on p. 747 to discuss valvular tissue engineering on bioresorbable scaffolds and comments:

> "In most cases, ***the valve candidates have been implanted in the pulmonary, rather than the aortic position, because the degrading scaffold cannot bear left ventricular pressures*** before new tissues being regenerated." (Emphasis added)

In addition to the issue of the structural stability of tissue engineered heart valves, Vesely remarks 'failed experiments' using resorbable scaffolds were not reported:

> "Personal communications with the principal investigators, however, confirm that ***fibrosis, retraction, and incompetence have hampered the progress of valves based on resorbable matrixes.*** No good histological pictures of failed valves appear in the literature, and thus little can be learned from the 10 years of experience with this approach."

Applicants therefore conclude that, contrary to the Examiner's assertions, a person of ordinary skill in the art would not have expected a tissue engineered heart valve to sustain the harsh hemodynamic conditions present in the heart, especially in the absence of any external support. The durability and strength of Applicant's tissue engineered valve is therefore unexpected and hence unobvious.

Applicants submit the Examiner has failed to demonstrate a *prima facie* case of obviousness for the following reasons.

1) The DE 19919625 publication is not prior art under 35 U.S.C 102(a) because it describes the Applicant's own work;

2) The combination of the Hoerstrup and Arru references fails to teach or suggest every limitation of Applicant's claimed invention;

3) Arru teaches away from Applicant's invention, and

4) Success in the field of tissue engineered heart valves is highly unpredictable hence non-obvious

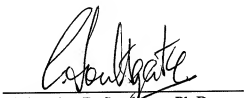
For the above reasons, Applicants respectfully request that the rejection of the claimed invention in view of Hoerstrup and Arru under 35 U.S.C § 103 be withdrawn.

CONCLUSION

In view of the above, reconsideration and allowance of this application are now believed to be in order, and such actions are hereby solicited. If any points remain in issue which the Examiner feels may be best resolved through a personal or telephone interview, the Examiner is kindly requested to contact the undersigned at the telephone number listed below.

The USPTO is directed and authorized to charge all required fees, except for the Issue Fee and the Publication Fee, to Deposit Account No. 19-4880. Please also credit any overpayments to said Deposit Account.

Respectfully submitted,



Christopher D. Souffgate, Ph.D.
Registration No. 54,875

SUGHRUE MION, PLLC
Telephone: (202) 293-7060
Facsimile: (202) 293-7860
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Heart Valve Tissue Engineering

Ivan Vesely

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Custom Design of the Cardiac Microenvironment With Biomaterials

Heart Valve Tissue Engineering

Engineering a Small-Diameter Artificial Artery

Engineering Myocardial Tissue

Regenerative Cardiomyocytes for Cardiovascular Tissue Engineering

Richard T. Lee, Guest Editor

Heart Valve Tissue Engineering

Ivan Vesely

***Abstract*—**Tissue-engineered heart valves have been proposed by physicians and scientists alike to be the ultimate solution for treating valvular heart disease. Rather than replacing a diseased or defective native valve with a mechanical or animal tissue–derived artificial valve, a tissue-engineered valve would be a living organ, able to respond to growth and physiological forces in the same way that the native aortic valve does. Two main approaches have been attempted over the past 10 to 15 years: regeneration and repopulation. Regeneration involves the implantation of a resorbable matrix that is expected to remodel in vivo and yield a functional valve composed of the cells and connective tissue proteins of the patient. Repopulation involves implanting a whole porcine aortic valve that has been previously cleaned of all pig cells, leaving an intact, mechanically sound connective tissue matrix. The cells of the patients are expected to repopulate and revitalize the acellular matrix, creating living tissue that already has the complex microstructure necessary for proper function and durability. Regrettably, neither of the 2 approaches has fared well in animal experiments, and the only clinical experience with tissue-engineered valves resulted in a number of early failures and patient death. This article reviews the technological details of the 2 main approaches, their rationale, their strengths and weaknesses, and the likely mechanisms for their failure. Alternative approaches to valvular tissue engineering, as well as the role of industry in shaping this field in the future, are also reviewed. (*Circ Res*. 2005;97:743-755.)

Key Words: cardiac valves ■ tissue engineering ■ review ■ acellular matrix ■ scaffold

Every year, more than 100 000 US patients need to have their dysfunctional or diseased valves replaced with a prosthetic valve. Where there is a need, there is a technological solution. The heart valve industry is the US is vibrant and healthy, enjoying a growth in the market of 5% per year, selling roughly 300 000 valves worldwide.[1] Worldwide sales were $910 million in 2002 and are most likely past the $1 billion mark in 2005. Faced with such tremendous market opportunities, many companies, clinicians, and scientists alike have taken serious interests in developing a new type of heart valve that can potentially revolutionize the industry and the practice of medicine. A tissue-engineered valve promises to be a living implant with a potential to grow and last a lifetime, like most native valves do. Rather than being a device that palliates a disease, it promises to be curative: a living replacement for a diseased component of our physiology.

As reviewed here, however, tissue engineering has promised much more than it has delivered. Indeed, heart valve tissue engineering has been hyped to such an extent that there is roughly 1 review article for every 4 true research reports published in this field. A simple PubMed search on "heart valve tissue engineering" reveals 164 citations since 1995, 32 of which are actually review articles.[2–33] This article, part of

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From The Saban Research Institute of Children's Hospital Los Angeles, Keck School of Medicine, University of Southern California.

The author has in the past, and is now, providing expert opinion on heart valve litigation, including tissue-engineered valves.

Correspondence to Ivan Vesely, PhD, The Saban Research Institute of Children's Hospital Los Angeles, Keck School of Medicine, University of Southern California, 4650 Sunset Blvd–MS137, Los Angeles, CA 90027. E-mail ivesely@chla.usc.edu

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a series of reviews on tissue engineering in *Circulation Research*, intends to examine what has been accomplished and what is realistically possible in the coming years. Patients and physicians alike are beginning to ask "When can I get a tissue-engineered heart valve?" Indeed, even the American Heart Association Web site has an article entitled "Tissue-engineered valves give diseased hearts new life,"[34] suggesting that clinical use of tissue-engineered valves is around the corner. The American Heart Association document fails to report the clinical outcomes of the cited experiment: that the technology has largely failed and that the claims of the surgeon have been discredited. This review thus aims to present a much more critical report on the real progress in this field, pointing out the specific mechanism by which tissue-engineered heart valves have failed in clinical and animal experiments.

As a discipline of its own, tissue engineering is surprisingly old. The term "tissue engineering" was coined by Fung in October 1987 at a National Science Foundation workshop in Washington, DC.[35] The different approaches to tissue engineering appeared to originate independently as early as the 1960s, when advanced tissue culture technologies were used to propagate skins cells.[36] In the mid-1970s, the work of Rheinwald and Green at the Massachusetts Institute of Technology set the stage for skin grafting with sheets of cultured, autologous keratinocytes.[37] Tissue engineering has had a number of definitions, both simple and complex. One of the simplest is that found in *The Biomedical Engineering Handbook*[38] (D. Williams, personal communication, 2005) and is stated as follows:

> The application of scientific principles to the design, construction, modification, growth and maintenance of living tissue.

A more complex definition is given in a World Technology Panel Report[39] funded jointly by the NIH, the Food and Drug Administration (FDA), and other government agencies, in which tissue engineering was defined as:

> The application of principles and methods of engineering and life sciences to obtain a fundamental understanding of structure-function relationships in novel and pathological mammalian tissues and the development of biological substitutes to restore, maintain or improve tissue function.

For the purposes of this review, tissue engineering relevant to heart valves is defined as the manipulation of biological molecules and cells for the purpose of creating new structures capable of metabolic activity.

A new type of heart valve thus fabricated must, therefore, contain material of biological origin in a configuration that did not emerge naturally. Heart valves containing trace amounts of biological material, such as pannus overgrown, are not considered tissue-engineered valves. Valves consisting of inert materials covered with a cellular coating, developed for the purpose of improving the performance of the valve, could be considered tissue-engineered devices. Before exploring the universe of tissue-engineered valves, it is best to first review the spectrum of existing prosthetic valves based on biological materials.

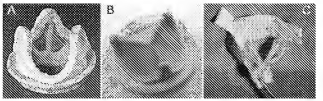

Figure 1. Images of a porcine bioprosthetic valve xenograft (A), bovine pericardial valve (B), and a human aortic valve allograft (C), also called a homograft. Both the porcine and bovine valves are treated with glutaraldehyde before implantation. The homograft is stored frozen and implanted without any other chemical preparation and often without any tissue type matching.

Bioprosthetic Valves

There are generally three types of bioprosthetic valves available commercially: (1) porcine xenograft valves, (2) bovine pericardial valves, and (3) allograft or homograft valves (Figure 1). The porcine xenograft valve consists of an intact pig aortic valve that is preserved in low-concentration glutaraldehyde solution.[40] These valves are prepared by valve manufacturers in various configurations, such as with or without integrated sewing cuffs, to maximize either ease of implantation or effective orifice area. Occasionally, these valves are assembled from up to 3 separate segments of aorta and the associated cusp material in an effort to improve valve symmetry and hence perceived performance.[41] The bovine pericardial valve is fabricated from up to 3 separate pieces of glutaraldehyde-treated calf pericardium, affixed to a supporting stent and sewing cuff, in a configuration very similar to that of the porcine xenograft. Both the porcine and bovine valve tissues are crosslinked in low concentrations of glutaraldehyde to reduce their antigenicity and to stabilize the tissue against the proteolytic degradation that would otherwise occur following implantation into the recipient. Both types of valve tissues are also treated with various other chemical agents to minimize their propensity to calcify over the duration of implantation and hence improve their longevity.[42] The homograft valves are intact human valves obtained from organ and tissue donors, usually stored cryopreserved as entire aortic or pulmonary roots, and trimmed to size and shape before implantation in the recipient.[43,44] These 3 types of valves are used primarily in the aortic and pulmonary positions and are occasionally inverted and used to replace the mitral valves. Mitral valve repair is used more frequently than mitral replacement during the first surgery to address a dysfunctional mitral valve. In addition to these 3 "device-related" approaches, there are also completely surgical approaches to reconstruct valves, making use of autologous or commercially available bovine pericardium to augment defective cusps or fabricate monocusp valves.[45] These highly varied approaches are used primarily in children who do not tolerate prosthetic devices nearly as well as adults do. The Ross procedure, the surgical removal of the autologous pulmonary valve and its reimplantation in the aortic position, is a hybrid approach that makes use of surgical reconstruction

of the aortic valve and a replacement of the missing pulmonary valve with a cadaveric homograft.[46] Although these surgical approaches often place biological tissues in new configurations, they are not considered tissue engineering, as there is little manipulation to the internal molecular structure for the purpose of enhancing their biological performance.

Why the Need for a Tissue-Engineered Valve?

Some may argue that there is not much of a need for tissue-engineered valves, because conventional valve technology is very mature with well-described performance criteria. Indeed, the original motivation for the continued development of prosthetic valve technologies appears to have lessened considerably with the improvements in surgical approaches and hence surgical outcomes. In the early days of bioprosthetic valve development, durability and hence longevity, was the main motivating factor. The reason why durability was the main object of research and development is that prosthetic valves are meant to be implanted once and should last the life of the patient. Historically, the main complication associated with the use of prosthetic valves has been operative mortality during prosthetic valve replacement surgery. Whereas the mortality during the first surgery—the surgery to replace the disease native valve—is often less than 1%, the second surgery is considerably more risky, with repeat operations having mortality as high as 20%, varying highly across institutions.[47,48] With improvements in surgical technique and technologies, reoperative mortality has been reduced considerably during the past decade. Many surgeons now view surgical mortality during reoperation to be lower than the cumulative risk of thromboembolism associated with the use of mechanical valves (approximately 4% per patient-year[49,50]) and now opt for repeated use of bioprosthetic valves in their patients. Indeed, the durability of the Edwards pericardial valve, perceived by many to be the most durable bioprosthesis, is close to 20 years[51,52] and almost equivalent to the aortic valve homograft which, because of its excellent longevity, is considered by many to be the gold standard.[43]

Because of the relatively good performance of current generation prosthetic valves, and the excellent quality of life they provide, both surgeons and valve manufacturers have become quite conservative. Surgeons are often reluctant to switch from a proven valve to a new device, and manufacturers are wary of introducing a new product that will have clinical performance that is worse than its current product. A number of clinical failures of new products have all but stifled innovation in conventional heart valve technologies. The failure of the Carbomedics Photofix-α valve (cusp abrasion and perforation),[53] the Medtronic Parallel mechanical valve (thromboembolism),[54] and the St Jude Silzone coating (tissue necrosis and perivalvular leak)[55,56] have made the valve industry highly aware of the possibility that innovation can breed disaster. Most recently, Edwards Lifesciences suspended the clinical trial of its catheter-deployable valve[57] because of problems with delivery and anchoring of the valve in the aorta of the patient.[58]

In the world of conventional valve technologies, the bar for the adaptation of a new valve is very high. Current bioprosthetic valves have a lifespan of 15 to 20 years,[59] highly predictable failure patterns[60] that can be managed, and negligible early complications. A tissue-engineered valve will, therefore, need to compete in this arena, where change is slow and methodical, often taking a generation to establish. Indeed, the Edwards valve has increased its market share steadily over the past 20 years, primarily because of clinical reports on its very good long-term performance.[51]

There may not be any immediate need for a tissue-engineered valve in the adult patient. Given the good service life that conventional valves offer, experimental use of new tissue-engineered valve concepts is questionable. The more realistic option for the use of tissue-engineered valves in the near future is in the pediatric population. The performance of the many surgical corrections for valvular defects is highly variable and depends on the age of the child.[61] Allograft valves are difficult to obtain for children because they require the death of other children of similar size. Surgical reconstruction and monocusp valves tend to fibrose and contract early.[62] In the child, there is a need for new materials and new approaches and thus an opportunity for tissue-engineered valves. Unfortunately, the market size for pediatric products is very small, less than 10% of the adult valve market, and thus not commercially viable. Accordingly, established heart valve manufacturers expend relatively few resources developing tissue-engineered valve technologies (neither Medtronic nor Edwards Lifesciences has a significant research program in heart valve tissue engineering; Medtronic and Edwards Lifesciences, personal communications, 2005). Clinical use of tissue-engineered valves will thus most likely happen first in pediatric hospitals on an ad hoc basis and will augment the portfolio of surgical options currently available to treat these very challenging patients.

Three Approaches to Valvular Tissue Engineering

Perhaps the first examples of heart valve tissue engineering were the experiments with seeding cells on otherwise inert substrates. This approach has been relatively successful in vascular grafts[64] but not at all for cardiac valves. Compliant synthetic materials have failed as valve leaflet substitutes, not because of thromboembolism but because of material fatigue, often related to microcracks, plasma protein insudation, and subsequent mineralization.[65] Because of these fundamental material issues, glutaraldehyde-preserved biological matrixes have dominated the valve field. Because of the residual toxicity of glutaraldehyde, glutaraldehyde-treated valve tissues have remain cell free. For reasons not completely clear, they do not induce thromboembolism and remain an essentially passivated structured in the blood stream, which does not elicit any adverse reaction.

Perhaps the first example of heart valve tissue engineering came out of the University of Vienna in 1991, when Grimm et al presented their success in inducing endothelium to grow on glutaraldehyde-fixed bovine pericardium.[66] Sustaining cells on the otherwise toxic pericardial tissue was made possible by inactivation of the aldehyde by crosslinking with L-glutamic acid before cell seeding. For unknown reasons, this technology was never implemented clinically and the last article on this topic was published in 1993.[67]

The approaches that have been sustained by investigators around the world can be grouped into the following two areas: (1) decellularization of xenogenic tissues followed by cell seeding or direct implantation and (2) use of bioresorbable synthetic scaffolds. A third, less popular approach, involves fabrication of cell matrix constructs by way of polymerization and cell entrapment.

Acellular Matrix Xenograft

This is perhaps the oldest approach to mainstream valvular tissue engineering, the first reports being patents by Brendel and Duhamel of the University of Arizona, Tucson, filed in 1984,[68] and by Klement et al[69] from Toronto, filed in 1987. Both of these approaches claimed the much larger field of acellular matrix use for applications ranging from valves to vascular grafts to bone, teeth, ligament, and skin. Decellularization of heart valves has since been attempted all over the world. In the US and Canada, this approach was attempted by Vesely and Noseworthy[70] and Wilson and colleagues[71] very early on in this field and more recently by the thorough investigation of Hilbert et al.[72] In Europe, this approach has been used more widely by Dohmen et al of Berlin, Germany,[73,74] Steinhoff and colleagues of Rostok, Germany,[75] Stock and colleagues of Jena, Germany,[76] Haverich and colleagues from Hanover, Germany,[77] Weigel et al from Vienna, Austria,[78] Gittenberger-de Groot and colleagues from Leiden, The Netherlands,[79] Fisher and colleagues of Leeds, UK,[80] and Spina, Gerosa, and colleagues from Padua, Italy.[81] In Asia, the approach has been adopted by Hong and colleagues[82] and Ye et al[83] from Shanghai, China, and by Wu et al from Beijing, China.[84]

The rationale for this approach is the assumption that the antigenicity of xenogenic tissues originates in the cellular debris. Recall that porcine aortic valves need to be crosslinked with glutaraldehyde before implantation and that human allograft valves do not. Clearly, the immunohistocompatability mismatch between humans and pigs is far more severe than it is between unmatched human subjects. Pig cells express the gal-$\alpha^{1,3}$ epitope, whereas humans do not.[85] Indeed, human allograft valves are almost never matched to recipients, even though matching has been shown to have better long-term graft survival.[86] Lack of matching is apparently not too detrimental because homograft valves can last 15 to 20 years. It has been theorized that perhaps the valve is in a privileged location, in a high flow environment where monocytes and other immune system cells cannot readily attach. With all of these issues in mind, suggesting that cell-extracted porcine valves can exist in human patients, without any appreciable antigenic response, may sound quite reasonable. This approach also assumes that these acellular matrixes will become repopulated with recipient cells, either before or immediately after implantation in the patient. A fully repopulated matrix would thus become "invisible" to the host immune system, because it would be enveloped in an endothelial cell layer recognized as "self," and possibly remodeled by the invading cells, ultimately becoming self as the host cells lay down a new matrix in place of the degraded porcine matrix. This approach has been developed to the greatest extent by Goldstein et al[87] of CryoLife Inc, a company well known for its homograft valve cryopreservation business. CryoLife eventually brought the technology to clinical use in Europe,[88] unfortunately with disastrous results that are reviewed later in this article.

The typical approach to generating an acellular matrix tissue is to first break apart the cell membranes through lysis in hyper- and hypotonic solutions, followed by extraction with various detergents. The detergents used by most investigators include the anionic Sodium dodecyl sulfate, the zwitterionic CHAPS and CHAPSO, and the nonionic BigCHAP, Triton X-100, and Tween family of agents. The enzymes that have accompanied these detergent treatments have focused mainly on cleaving and removing the DNA that is part of the cellular debris. Because these enzymes can potentially degrade the useful matrix, enzyme inhibitors, such as trypsin inhibitor, can be used, although some have used trypsin-EDTA alone to decellularize the matrix.[89] It is important to note that the agents used for cell extraction can be quite detrimental to the matrix: they can degrade or denature the matrix proteins or leave toxic residues or residual charge, any of which can detrimentally affect mechanical function or cellular response. The parameters that the many investigators in this field have varied involve mainly the sequence of steps, the specific detergents to use, and the time duration of the various soaking periods. For example, the laboratory of Fisher in Leeds, UK,[80,90] uses a series of baths in PBS and hypotonic buffer, along with trypsin and nucleases, to decellularize the tissue. The entire extraction procedure can last up to a week. Mechanical testing is used most of the time to determine the mechanical integrity of the processed tissues. Parameters such as burst pressure or failure strength of test trips is compared with unprocessed controls.[80,91] In most cases, mechanical properties remain well preserved after cell extraction. Histological morphology, however, varies greatly from process to process, often showing a highly porous, locally collapsed microstructure, suggesting that there are features of the matrix that are not readily measurable using conventional techniques. The patents that describe the creation of these acellular matrixes are perhaps the largest collection in this field and number in the dozens, if not hundreds.

To seed or not to seed cells before implantation has become 1 of the important variables to consider in this approach. Perhaps the most disconcerting issues in this approach are reports that homograft valves not only fail to repopulate with recipient cells but actually become completely acellular within months of implantation.[92] This is of particular concern as the human aortic valve is expected to be the ideal matrix for repopulation: it has the right mechanical properties and is far less antigenic than porcine matrixes. Possibly in view of these pathological findings, or possibly for other reasons, a number of investigators have attempted to repopulate these scaffolds in vitro, in advance of implantation into animal models.[93] The CryoLife technology,[87] however, was implanted into patients without any prior cell seeding in vitro. Indeed, technologies attractive to the valve industry are those that do not involve handling of patient cells. It is far easier to get a new valve approved by the FDA as a device, if there is no reliance on cells contributing to its long-term durability.

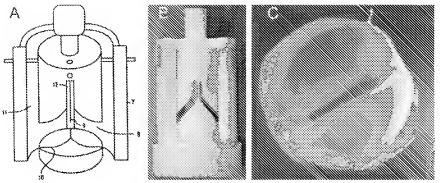

Figure 2. A, Image of the original mold by Tranquillo (patent 6,666,886). B, Photograph of real mold fabricated from Teflon. C, Image of bileaflet valve fabricated by casing a collagen or fibrin gel within this mold.

Bioresorbable Scaffold

This approach to valvular tissue engineering is perhaps the most conventional. The bioresorbable scaffold has been used in applications as varied as skin, bone, vessels, spinal chord, tendon, bladder, vagina, muscle, and solid organs like liver and pancreas. The concept is simple: cells of a particular phenotype are seeded on a porous material, implanted in the body, and are expected to generate the organ of interest as the scaffold degenerates. The scientific rationale for this approach is unclear and based primarily on empirical evidence that it appears to work in certain applications. Perhaps the oldest most successful application of this approach is tissue-engineered skin, dating back to the late 1970s.[94] This approach launched the tissue-engineering industry and remains the most, if not the only, successful product.[35]

The original scaffolds for heart valves were borrowed from skin: polylactic and polyglycolic acid and copolymers thereof.[95] These materials have been largely abandoned for heart valve applications because they were too stiff, and newer, more compliant materials like polyhydroxyalkanoate, have been used.[96] Natural scaffolds, like small intestine submucosa have also been used for valvular matrixes.[97] In most cases, the valve candidates have been implanted in the pulmonary, rather than the aortic position, because the degrading scaffold cannot bear left ventricular pressures before new tissues being regenerated. Besides the option of scaffold material and shape of the leaflets, the other variable is the decision of whether to preseed in vitro or not and, specifically, how to do it.[93] As discussed above for the acellular matrix valve, preseeding in vitro appears to yield better in vivo results.

Collagen-Based Constructs Containing Entrapped Cells

This is perhaps the least explored area and the one that involves the greatest amount of engineering. This approach is based on the relatively old observations that cells entrapped in collagen gels contract and compact the gels, increasing the density of the collagen many fold.[98–100]

The principle involves first mixing soluble, fibrillar collagen with the appropriate cells. After the collagen–cell mixture is neutralized, soluble collagen reassembles into fibrils and a gel is created. Cells become entrapped within the collagen gel and begin to interact with the collagen fibrils and contract the matrix, excluding water.[98,99,101] In many ways, this in vitro contraction mimics wound healing in vivo.[102,103] When the gel is mechanically constrained, the collagen fibrils align in the direction of constraint,[101] and a highly aligned, compacted collagenous construct can thus be fabricated.

One of the early applications of this technology has been as blood vessels,[105] where the fabrication of collagen tubes is relatively straightforward. All that is required is a rod affixed centrally within the lumen of a tube, creating an annular space that is filled with the collagen–cell mixture. Once the mixture gels, it begins to contract around the central rod and peels off the inner surface of the tube. At that point, the rod with its adherent gel coating can be removed from the tube. Because the gel coating on the rod cannot shrink in circumference, it compacts only through its thickness and along the length of the rod. Collagen fibrils thus align circumferentially, much like in the adventitia of a blood vessel.

A very logical extension of this approach to heart valves is to fabricate a mold for leaflets, contiguous with the aortic annulus. This is what has been done by the laboratory of Tranquillo et al.[106] Tranquillo developed an interesting mold with inner and outer parts that produced the shape of a bileaflet valve within a tube (Figure 2). Like the vessel wall, the valve leaflets also shrank along the direction of the tube, becoming shorter and developing an aligned fiber structure.[107]

The work in the laboratory of this author is a simplification of the original work of Tranquillo. Rather than forming complex 3D structures, we have focused on generating simple, 1D strings that could be used for surgical reconstruction of mitral valve chordae or for future use in more complex constructs. We began by selecting neonatal rat aortic smooth muscle cells as the experimental cell line, because they are well known for producing considerable amounts of matrix, particularly elastin.[108] We found empirically, however, that cells with highly varied phenotype can be extracted from the minced aortic tissues. Digesting these tissues with trypsin apparently liberates a mixture of cells, many of which are not highly contractile and thus unsuitable for use with directed collagen gel shrinkage. The best outcomes were obtained with cells isolated by the outgrowth method, in which tissue is minced into small pieces and plated onto to dishes. The most highly motile cells apparently grow out from these tissue fragments and can be amplified over several weeks of culture. Optimal shrinkage of the constructs occurred when these cells were added to the collagen suspension at a cell-seeding concentration of 10^6 cells/mL.[109] The collagen suspension consists of sterile acid-soluble type I collagen at an initial concentration of 2.0 mg/mL. For our application, the

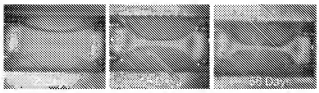

Figure 3. Images of collagen constructs during the shrinkage process, showing rapid compaction within a few days and more gradual, yet continuing, compaction over the next 8 weeks of culture. Reproduced from Shi and Vesely[109] with permission.

cell/collagen suspension is pipetted into rectangular wells of variable geometries with microporous holders at their ends. Like the inner mandrel of the tubular constructs, the anchors at the ends of the wells prevent longitudinal contraction and allow shrinkage to occur only transversely to the long axis of the wells. This gives rise to well-aligned collagen constructs with relatively high collagen fibril density (Figure 3).

After 8 weeks of culture, the collagen constructs have the typical nonlinear stress/strain curve of tendinous materials, an extensibility of 14%, a stiffness of 5 MPa, and failure strength of 1.1 MPa. Although the stiffness and strength are still about an order of magnitude lower than what is required, our constructs are already 10 to 100 times stronger than similar collagen-based materials fabricated previously.[110,111] Ultrastructural analyses have shown that the main reason for the good strength of our constructs is the very high collagen fibril density. Because the constructs are relatively simple 1D collagen bundles, they compact from 2 directions, producing an area shrinkage ratio that is greater than 99%.

In an effort to improve the strength of these constructs further, we have explored different sizes and aspect ratios, different materials for the anchors for these constructs, and different forms of application of external tension. For example, triangular-shaped holders that appear to channel the tension from the holder material to the construct lead to stronger constructs, as does the application of external forces. Dynamic loading, in particular, increases construct strength by a factor of 3. Although these constructs are clearly not ready for human use, they are nearing use in animal models. One point of concern is the use of rat collagen and rat cells, and efforts are underway to translate this technology from the rat to the sheep model, making use of sheep collagen and sheep cells. The core technology, however, is also being used in a more ambitious approach to develop a composite aortic valve cusp. This is discussed below.

Other Substrates in Early Development

The main problems with using reconstituted collagen as a substrate for tissue engineering is the observation that cells entrapped in collagen gels rapidly enter apoptosis[112] and synthesize matrix metalloproteinases. Whereas strategies for overcoming these phenomena have been attempted (ie, mechanical loading), use of collagen alone has thus not been widely embraced. A number of investigators are exploring cell adhesion and phenotype on thin flat films of candidate materials, as a prelude to using the bulk material as a scaffold. For example, Giachelli and colleagues have explored the use of chitosan, an interesting material derived from crustacean shells and used for wound dressings, and found that mixtures of chitosan and collagen work better than either alone.[113] Anseth and colleagues have used crosslinked polyvinylalcohol[114] and hyaluronan as a substrate for valve tissue engineering.[115,116] Rothenburger and colleagues from Muenster, Germany, have used collagen films as scaffolds for tissue engineering[117,118] and found that myofibroblasts and endothelium cocultured produced significant amounts of collagen and structural proteoglycans. Fibrin is being considered an alternative to collagen in valvular tissue engineering by a number of groups,[119] including that of Tranquillo.

History of Each Approach: Successes and Failures

Acellular Matrix Valve

Judging from the volume of literature, the acellular matrix valve has received the most interest. This is not surprising, because it seems to make the most sense from a biomechanical point of view. The aortic valve is only that: a mechanical structure designed to open and close with minimal pressure drop and reverse leakage.[120] What is unique to the aortic valve, compared with most other connective tissues, is that it does not require any metabolic activity or self-repair to provide a good service life. Recall that human aortic valve allografts (homografts) are transplanted without any tissue-type matching and consequently become acellular within a few months.[92] The homograft can last 20 years, essentially as a dead piece of tissue, free from any mechanical reinforcement by crosslinking agents. Indeed, it is remarkable that the homograft valves last longer than the glutaraldehyde-fixed porcine xenografts; there must be something about the microstructure and composition of the native aortic valve that makes it very resistant to mechanical fatigue. Indeed, many have studied the structural basis for this remarkable durability of the aortic valve.[120–143] Although it still remains unknown exactly what features of the native valve tissue give it such remarkable durability, the importance of its internal complexity is being appreciated more and more. Most likely, the presence of interconnected sheets of collagen, layers and tubes of elastin, highly nonlinear mechanics, anisotropy, and viscoelasticity endow the valve tissue with is unique longevity. Not knowing exactly which features are important, the logical approach is to duplicate all of them in a prosthetic device. Indeed, the use of intact porcine aortic valves has spawned the whole bioprosthetic valve industry. Early in its history, engineering analyses clearly demonstrated that the porcine xenograft, with its inherent structural complexity, is theoretically better than the pericardial valve[144] and should thus last much longer. Interestingly, this conventional wisdom ultimately proved to be quite wrong: a particularly good design of the pericardial valve (the Carpentier-Edwards valve) is remarkably long lived[51,52] and appears to be more durable than glutaraldehyde-fixed porcine valves.

Now it could be argued, of course, that a glutaraldehyde-fixed aortic valve, mounted on a stent, is hobbled somehow and its theoretical advantage over the pericardial valve disappears. That could very well be the case, because some studies have shown certain disadvantages of glutaraldehyde-

fixed aortic valve cusps relative to similarly prepared bovine pericardium.[145] The intact aortic valve may thus still have the upper hand when finally revitalized with freshly seeded cells. Regrettably, there have been few, if any successes, with this approach. Rather than happily repopulating the waiting matrix, the cells that come into contact with acellular valves have reacted badly and destroyed the intricate microstructure and its associated perfect mechanics. The reality of this, unfortunately, has come to light only recently. For almost 20 years, research groups around the world have independently tried various chemical processing approaches and published the successes in short-term animal models, but none led to any real clinical success.

The first reports that these protocols ultimately fail in long-term animal studies were only anecdotal at first, shared among scientists during personal discussion at the various heart valve conferences. No negative results were actually published until Hilbert, a well-recognized valve scientist working at the laboratories of the NIH, did the "ultimate" comparative study of various extraction protocols in a long-term sheep implantation study.[146] Hilbert copied the protocols of a number of investigators and implanted 2 sets of differently processed valves into the sheep model, as pulmonary artery interposition grafts. After 20 weeks of implantation, the valves were explanted and examined grossly and histopathologically. With some variation between treatment protocols, all valves underwent considerable tissue overgrowth and infiltration with inflammatory cells. There was also some evidence of aneurismal dilatation. The second such report came from the laboratory of Stock,[147] who reported similar complications with his valves, even though these valves were seeded with cells before implantation and were thus more "ready" for implantation than the valves prepared by Hilbert.

It could be argued, however, that the sheep model was destined for failure and that the acellular xenograft approach would work in the human condition. The reason for this argument is that it has become clear over the past decade that the sheep model generates an exuberant fibrotic response to valve implants. Valves that are implanted in sheep overgrow rapidly with fibrotic tissue, certainly much more than they do in humans.[53] Indeed, a painful lesson learned in this area was experienced by Sulzer Carbomedics, whose Photofix-α pericardial valve developed severe abrasions to the leaflets against the sewing cuff during its clinical trials in the mid-1990s. The reason for the leaflet abrasion was attributable to a very specific design flaw, which was not anticipated.[53] Indeed, this flaw did not manifest in the preclinical sheep studies because the sheep rapidly covered the sewing cuff with pannus and thus protected the delicate valve leaflets from the more abrasive sewing cuff material. It could be thus argued that testing acellular matrix valves in the sheep model is a waste of time and destined to fail, because of this severe fibrotic response.

Perhaps this was the motivation behind the use of the CryoLife Synergraft in patients directly, without any reported long-term animal studies, something quite unusual for the heart valve field. The CryoLife approach was similar to the others: the removal of cellular antigens through dissolution and extraction.[87] Unlike the somewhat harsh chemistry of the original Brendel and Duhamer approach,[68] and most of those who tried to improve on it, CryoLife favored a more gentle approach that has not been revealed publicly but most likely involves multiple freeze-thaw cycles, cell lysis through variation in osmotic pressure, use of enzymes such as DNase, and prolonged extraction in aqueous solutions. The CryoLife product was thus expected to be the least affected and the most mechanically sound acellular matrix. In October of 2000, CryoLife Inc obtained CE Mark approval to sell the Synergraft product in Europe[88] to a limited group of patients who had few alternatives: the neonate with congenital valvular malformations.

Although the numbers are not widely reported, a number of patients in Vienna, Austria, received these valves. Within a few weeks to months, many of these children began to experience serious valvular complications.[148] Although some remained apparently unaffected, many valves became highly regurgitant, and some children died. CryoLife rapidly withdrew the product from the market and disclosed the negative clinical outcomes at the 2004 Florence Heart Valve meeting. In their 2003 article, Simon et al[148] reported that 1 patient (aged 7 years) died 7 days after implantation of the valve, 1 patient (aged 9 years) died 6 weeks after surgery, and 1 patient (age 2.5 years) died 1 year after surgery. Death resulted from various cardiac complications related to inflammation, valve rupture, and stenosis. One patient was reoperated on 2 days after the primary surgery, in view of what happened to the others. All valves showed severe inflammation, both inside and out, fibrosis, encapsulation, perforation, and deterioration of the leaflet tissues. Grossly, this reaction was not markedly different from that shown by Stock and colleagues[147] in the sheep model. The sheep model was thus not to blame; it was the matrix itself after all. Apparently, there is some abnormal signaling generated by the acellular matrix that induces the invading cells to remodel, fibrose, and contract what otherwise appears to be a perfect, mechanically sound valve matrix. The exact reasons for this remain unclear.

Although its first clinical use was disastrous, proponents of this approach remain undeterred. Apparently, another variant of this approach is doing well in Berlin, Germany, and is being implanted by Dohmen, Konertz, and colleagues[73,74] with 50 implants in patients for more than 2 years. In this series, 1 patient died postoperatively, 2 required reoperation for valve-related complications, and the rest of the patients appear to be doing fine. The published short-term results in adults (17 to 70 years; mean, 46 years) therefore appear to be good, and the surgeons are satisfied with this technology. Personal reports obtained at the Society for Heart Valve Disease Meeting in Vancouver in June of this year, however, indicate that a number of their patients have indeed died from valve-related complications. In their article,[73] the authors also disclose that Konertz and Dohmen are shareholders of Auto-Tissue GmbH, the company that produces these valves.

Resorbable Scaffold

The failure of this approach is much less dramatic, as apparently none of these scaffolds have been tried in patients.

Interestingly, the main and still dominant reason for favoring the bioresorbable polylactic/polyglycolic acid (PLA/PGA) scaffold approach is the FDA approval of the materials for implantation in the US, as bioresorbable sutures. Although this may be true, it most likely does not lessen the regulatory burden of a device constructed from these materials. Valves are life-sustaining class III devices and must be taken through the full set of preclinical and clinical trials before market approval. Perhaps it is thought that as limited-use surgical materials, valves fabricated from these matrixes could be used in limited volumes by surgeons directly, as part of an investigative study in their hospital. Perhaps the attractiveness of this approach is the legacy of Robert Langer who pioneered the resorbable matrix approach in the artificial skin product and launched the tissue engineering industry. Perhaps the entire approach is flawed. Jeff Hubbel, a noted chemical engineer with expertise in developing materials with controlled release chemistry noted recently at a conference that biological matrixes do not degrade through hydrolysis, but rather through proteolysis. Accordingly, he has been engineering his matrixes to be cleaved by enzymes.[149] Hydrolysable matrixes therefore are clearly not biomimetic and their use as biodegradable substrates should thus give rise for concern. Matrixes cleavable by proteolytic enzymes, however, are also far from clinical reality and may indeed experience the same fate as the others.

The fate and the failure mechanism of the bioresorbable matrix approach to heart valves, unfortunately, is less clear than it is for the acellular xenograft. Like for the acellular matrix valves, there have been no clear reports of "failed experiments." Personal communications with the principal investigators, however, confirm that fibrosis, retraction, and incompetence have hampered the progress of valves based on resorbable matrixes. No good histological pictures of failed valves appear in the literature, and thus little can be learned from the 10 years of experience with this approach. Each group working in this field appears to publish promising short-term results with a particular matrix material and yet abandons it in favor of a more promising one with no report of how the old material fared. What remains unclear to most of the scientific community is specifically how the previous approach failed. Although PLA/PGA[95] was abandoned by the pioneers in this field—the group of Vacanti and Mayer of Harvard/Children's Hospital—in favor of a polyhydroxyalkanoate, a better, more compliant material,[96] other groups around the world continue to work with PLA,[150] most likely because it is the only FDA-approved, clinically popular material. As the various research groups working in this field continue to use resorbable materials, the questions that remain can be unpleasant: Are PLA/PGA and their variants inappropriate materials, or have they simply been used improperly by the first groups? Are the other groups going to see the same results and waste a lot of time? What is the responsibility of the research community in helping others avoid approaches that failed and perhaps choose a better path? In an environment where the prize is a piece of a $1 billion annual market, competition for attention and credibility is clearly felt at scientific meetings. Reporting about failures, rather than successes, also does not make for exciting reading and negative results seldom get published in mainstream journals. The ability for scientists to learn from the mistakes of the past is therefore quite limited.

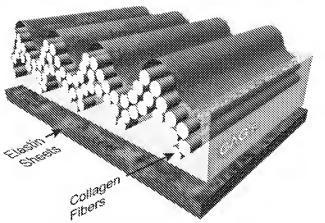

Figure 4. Schematic diagram of the multilayered configuration of an aortic valve cusp, showing the location of the Collagen fibers in the fibrosa, the elastin sheets in the ventricularis, and the GAG-rich matrix of the watery spongiosa.

Hybrid Approaches

A possible alternative to the acellular valve and the bioresorbable matrix approaches is the fabrication of complex structures by manipulating biological molecules. With sufficient fidelity, one could potentially fabricate structures as complex as the aortic valve cusps. This approach is a derivative of that pioneered by Tranquillo,[100] except that the directed collagen shrinkage method is simplified to a process that forms essentially 1D strings of collagen. These collagen fiber bundles can then be used as building blocks for the development of a composite aortic valve cusp.[151] Whereas collagen structures in heart valves can be found in the form of sheets,[152] most of the load-bearing components of the valve cusp are relatively thick, dense collagen fiber bundles. Having developed this technology already[109,153] the next step was to develop the other components required to make this approach work.

The aortic valve consists of the three basic building blocks of all connective tissues: collagen fibers, elastin sheets, and glycosaminoglycan matrix schematically arranged as shown in Figure 4. The collagen exists to bear tensile load and provide some ultimate stiffness and strength to the valve, so that it can withstand diastolic loads.[125] The elastin matrix exists to return the collagen structures back to their resting states between loading cycles,[124,126] and the glycosaminoglycans likely maintain hydration and the intrinsic viscoelasticity of the tissue.[123,142,154] For the latter component, hyaluronan was chosen as the working material.

Hyaluronan is a glycosaminoglycan polymer with a repeating disaccharide structure (glucuronic acid–b1,3-*N*-acetylgalactosamine-b1,4-)*n*, where *n* can reach 25 000 or more. In solution, hyaluronan forms large, random coil structures that occupy large solvent volumes. When constrained within a matrix, like a collagen network, hyaluronan exerts a swelling pressure that traps water, gives tissues compressive resistance, and imparts viscoelastic properties.

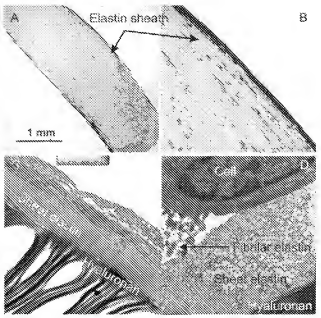

Figure 5. Histological images of longitudinal sections of a collagen construct showing the new elastin sheath under low (A) and high (B) magnification. Transmission electron micrographs of hyaluronan gels with an adherent elastin sheet just underneath the cell layer, shown under low (C) and high (D) magnification. Panel B reproduced from Shi and Vesely[165] with permission; panel C modified from Ramamurthi and Vesely[166] with permission.

Hyaluronan also plays a role during embryonic cardiac development as invading cells transform "cardiac jelly" into specialized cardiac structures, like the myocardium and the cardiac valves.[155] Hyaluronan is exceptionally biocompatible.[156–158] Unlike collagen that has both tissue- and species-specific markers, hyaluronan exhibits structural homology across species. For example, hyaluronan made by bacteria is the same as hyaluronan made by humans. Because of its interesting viscoelastic properties and its broad biocompatibility, hyaluronan has been used in a number of clinical applications and also as a scaffold for heart valve tissue engineering[115] by others.

The formulation of hyaluronan gels used in our laboratory is based on a modification of a previously patented protocol.[159] Details of the preparation are published elsewhere,[160,161] but in brief, the process involves mixing commercially available sodium salt of long chain hyaluronan with 1 mol/L NaOH at low temperatures and crosslinking with divinyl-sulfone. These gels can have a stiffness as high as 30 kPa, similar to that of the elastin structures of the aortic valve cusps, and thus can form highly hydrated, elastic sheets. It is expected that appropriately cast sheets of crosslinked hyaluronan can find use in the central spongiosa layer of the aortic valve cusp.

Two spurious findings occurred when working with the collagen constructs and the crosslinked hyaluronan; both stimulated the formation of elastin sheets. Elastin sheets formed spontaneously around the periphery of the collagen constructs[153] and on hyaluronan substrates texturized through dehydration and texturization with ultraviolet light[162] (Figure 5). Whereas the growth of elastin sheets on the collagen constructs is straightforward, growth of elastin sheets atop the hyaluronan gels has been difficult to reproduce. The success of this technique appears to be highly dependant on the specific cell type, gel formulation, and degree of surface texturization by UV light irradiation. Standardization of these protocols is an ongoing process in our laboratory, and no animal implants have yet been done.

Stem Cells and Other Future Prospects

The cell is clearly an important component of the tissue-engineered heart valve. A very good review of the types of cells used in tissue-engineered valves is provided by Flanagan and Pandit.[9] Much of the work identifying the phenotype of interstitial cells in the aortic valve has been done outside the US, in the laboratories of Yacoub,[163,164] Gerosa,[165] and Boughner.[166] Which cell to use for seeding scaffolds remains unclear. Stem cells and various other progenitor cells are being increasingly used in tissue-engineered valve applications. The 2 main cell types are mesenchymal stem cells and circulating endothelial progenitor cells. These cells are harvested from either experimental animals or patients, expanded in culture, and then seeded on the various valve leaflets substrates, be they the acellular matrix valves or the resorbable scaffolds. From presentations at recent conferences, all of these studies are very preliminary, showing no real differences in histological morphology over conventional interstitial cells used in previous studies.

Many investigators believe that cells are "smart," somehow recognizing the substrate and behaving in the appropriate way. The fibrotic overgrowth and failure of valvular matrixes described above clearly points to the contrary. With the emergence of stem cell science, many investigators believe that stem cells are going to be the "silver bullet" and are going to be smarter and act appropriately on the substrates. Embryonic stem cells, when injected into infracted hearts, have not behaved "smartly," did not rebuild the damaged myocardium, and, instead, created calcific deposits or teratomas.[167] There is no evidence that undifferentiated stem cells will behave in a more intelligent way on valvular substrates. Most likely, considerable effort will need to be expended to differentiate embryonic stem cells along a valvular lineage in advance of seeding on valvular substrates, before the promise of stem cells can be realized in this field.

Stem cell, however, are clearly the wave of the future. Considerable evidence exists in the literature that matrixes implanted without cells resorb, fibrose, and fail to produce any clinical benefit, whereas matrixes seeded with relevant cells incorporate and offer therapeutic benefit. The work of Atala in the field of urology is perhaps the best example of cell-seeded matrixes providing real clinical benefit to patients in augmenting large soft tissue defects.[168] Stem cells, once differentiated to the proper end point, are expected to provide a broader source of autologous cell lines, along with the appropriate matrixes, for therapeutic use in the cardiovascular field.

The use of autologous cells, cell expansion, preimplantation culture, and other cell "management," however, is not the preferred approach in the medical device and therapeutics industry. Because of regulatory and cost issues, a tissue-engineered valve that does not require any cell management is the preferred choice for medical device companies. This is

the reason for which CryoLife and originally St Jude and Advanced Tissue Sciences and currently Medtronic have pursued the acellular matrix approach; it can be qualified, manufactured, and sold as a device, a far simpler approach than the biological or combination product that most other tissue engineering therapies are likely to follow. The fact that the CryoLife approach failed, however, has not deterred others from trying it. The literature clearly shows that the majority of research projects prefer the acellular matrix approach, particularly now with the greater promise of stem cells.

Conclusions

Perhaps the most often asked question by outsiders is, "When will tissue-engineered valves be ready for clinical use?" Given that heart valve tissue engineering is a field already almost 20 years old, and the only real clinical experience (the use of the Synergraft technology in children) has been disastrous, it may take another 20 years before the many complex challenges are finally solved. Although occasional experimental use of tissue-engineered valves in children will likely occur sooner, because children with congenital outflow tract abnormalities have few options, it will take 20 years to demonstrate that the long-term performance of tissue-engineered valves is comparable or better than conventional glutaraldehyde-treated porcine xenografts or pericardial valves. Children and adult patients with no option for conventional treatment will thus continue to serve as the proving ground for tissue-engineered solutions for cardiovascular defects for the foreseeable future, hopefully with less tragic consequences.

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Bioengineering Challenges for Heart Valve Tissue Engineering

Michael S. Sacks,[1] Frederick J. Schoen,[2] and John E. Mayer, Jr.[3]

[1]Department of Bioengineering, Swanson School of Engineering, University of Pittsburgh, Pennsylvania 15219; email: msacks@pitt.edu

[2]Departments of Pathology, Brigham and Women's Hospital and Harvard Medical School, Boston, Massachusetts 02115

[3]Department of Cardiac Surgery, Children's Hospital of Boston and Harvard Medical School, Boston, Massachusetts 02115

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Abstract

Surgical replacement of diseased heart valves by mechanical and tissue valve substitutes is now commonplace and enhances survival and quality of life for many patients. However, repairs of congenital deformities require very small valve sizes not commercially available. Further, a fundamental problem inherent to the use of existing mechanical and biological prostheses in the pediatric population is their failure to grow, repair, and remodel. It is believed that a tissue engineered heart valve can accommodate many of these requirements, especially those pertaining to somatic growth. This review provides an overview of the field of heart valve tissue engineering, including recent trends, with a focus on the bioengineering challenges unique to heart valves. We believe that, currently, the key bioengineering challenge is to determine how biological, structural, and mechanical factors affect extracellular matrix (ECM) formation and in vivo functionality. These factors are fundamental to any approach toward developing a clinically viable tissue engineered heart valve (TEHV), regardless of the particular approach. Critical to the current approaches to TEHVs is scaffold design, which must simultaneously provide function (valves must function from the time of implant) as well as stress transfer to the new ECM. From a bioengineering point of view, a hierarchy of approaches will be necessary to connect the organ-tissue relationships with underpinning cell and sub-cellular events. Overall, such approaches need to be structured to address these fundamental issues to lay the basis for TEHVs that can be developed and designed according to truly sound scientific and engineering principles.

Contents

INTRODUCTION

Surgical replacement of diseased heart valves by mechanical and tissue valve substitutes is now commonplace and enhances survival and quality of life for many patients. However, repairs of congenital deformities require very small valve sizes that are simply not commercially available. Further, in pediatric applications, growth of the replacement valve is essential to eliminate the need for reoperations as the patient grows. There are a variety of devices available for replacement of cardiac valves, but all current devices have significant limitations that result in a continuing risk for morbidity and mortality (1).

*Erratum

The three predominant types of prosthetic devices now utilized for valve replacement include mechanical valves (MV), bioprosthetic heart valves (BHV), and cryopreserved homograft valves (CHV). Although valve replacement with these devices generally results in an improvement compared with the natural history of the untreated valvular heart disease, each type of valve replacement device has particular problems. Mechanical valves are thrombogenic and thus require lifelong anticoagulation, which reduces (but does not eliminate) the risk of valve thrombosis and embolization of thrombotic material (2). These valves are also much more susceptible to infection, and once established, infection is extremely difficult to eradicate without replacing the prosthesis (3, 4). The two primary processes underlying poor BHV durability are leaflet mineralization, with or without leaflet tearing, and mechanical fatigue (i.e., noncalcific tearing) (1, 5, 6). The majority of degenerated valves have both calcification and leaflet defects, while stenosis due to calcification or mechanical damage alone occurs much less frequently (5). High levels of calcification generally coincide with regions of high flexure or experience localized mechanical forces, such as the commissures and basal attachment (5, 7, 8). In addition, isolated noncalcific disruption of BHV has been found in clinical explants (9–11). Structural degradation without calcification has also been observed in nonchemically treated cryopreserved allograft tissues (5), underscoring the importance of understanding the fatigue properties of the collagen architecture. In general, homograft valves have advantages and disadvantages similar to BHVs and have the additional significant problem of limitations of supply. These valves ultimately fail because collagen damaged during function cannot be repaired.

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The essential design criteria for a replacement heart valve were originally articulated in 1962 by pioneering heart surgeon Dr. Dwight Harken (12) and extended for the tissue engineered heart valve (TEHV) as (*a*) it must be nonobstructive, and its closure must be prompt and complete; (*b*) it must be made up of a nonthrombogenic living tissue that lasts the lifetime of the patient; (*c*) it must provide an ongoing remodeling process that facilitates a homeostatic functional state and repair of any cumulative injury; and (*d*) it must accommodate the somatic growth of the recipient. *Erratum

In particular, such a valve would be useful in the treatment of the approximately 20,000 children with congenital heart disease born in the United States each year, especially those with valvular disease. In this population, the anticoagulation required with mechanical valves is particularly dangerous, and tissue valve substitutes undergo accelerated calcification. Moreover, the placement of oversized valves extra-anatomically in the right ventricular outflow necessitates multiple subsequent surgeries for these children as valves and conduit devices repeatedly become stenosed over time.

Thus, a fundamental problem inherent in the use of existing mechanical and biological prostheses in the pediatric population is their failure to grow, repair, and remodel. Regardless of the design specifics of current prosthetic valve devices, none offers any potential for growth, and therefore pediatric patients requiring valve replacement will require reoperations to place larger devices to accommodate the growth of the patient (13). It is believed that a TEHV can accommodate many of these requirements, especially those pertaining to somatic growth. The purpose of this review is to provide an up-to-date overview of the field of heart valve tissue engineering, including recent trends, with a focus on the unique bioengineering challenges. This contribution also extends and updates previous related reviews (13a–c), with a focus on the unique bioengineering challenges inherent in cardiac valves and their function.

KEY STRUCTURE-FUNCTION CORRELATIONS IN THE CARDIAC VALVES

Because heart valves are a relatively specialized area of the research, we begin by briefly reviewing their structure and function. For a more detailed review of heart valve biomechanics and function, the interested reader is referred to some recent reviews (14–16). Heart valves are structural specializations of the cardiac tissues that ensure unidirectional blood flow through the heart. The heart valves open and close approximately 40 million times a year and 3×10^9 times over an average lifetime. Normal valves are free of obstruction in the open position and without regurgitation in the closed position. The motions of the heart valves during opening and closing are driven by mechanical forces exerted by the surrounding blood and heart. The ability of the heart valves to permit unobstructed forward flow depends on the mobility, pliability, and structural integrity of their delicate flaps, generally called leaflets [in the tricuspid valve (TV) and mitral valve (MV)] and cusps [in the pulmonary valve (PV) and aortic valve (AV)]. *Erratum

The AV, which is most frequently diseased, is most frequently used as a substitute valve (derived from animals or deceased humans), and is most widely studied, provides a paradigm for valvular structural specialization and tissue dynamics. The individual AV cusps attach to the aortic wall in a crescentic (or semilunar) fashion, ascending to the commissures (where adjacent cusps come together at the aorta) and descending to the basal attachment of each cusp to the aortic wall. Behind the cusps are dilated pockets in the aortic root, called sinuses of Valsalva, which bulge with each ejection of blood.

In the closed phase, when there is backpressure from the blood in the aorta, the AV cusps stretch and mold to fill the orifice. Coaptation of the AV cusps is maintained by a complex, highly differentiated, dynamic tissue macro- and microstructure, which we will describe below. The

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correlations of structure and function for the cardiac valves have recently been described in detail (14). PV structure is analogous to but less robust than that of the AV, consistent with the lower pressure environment. Heart valves are sufficiently thin to be nourished by diffusion from the blood bathing the valves, and normal leaflets and cusps have only scant and inconsistent blood vessels and nerves (16).

VALVULAR EXTRACELLULAR MATRIX

Heart valves must not only accomplish the second-to-second movements during the cardiac cycle described above but also must maintain sufficient strength and durability to withstand repetitive and substantial mechanical stress and strain over many years (and thus many millions of cycles). This is accomplished by a highly responsive, complex internal microarchitecture, which facilitates the substantial changes in size and shape of the valve cusps and leaflets that occur during the cardiac cycle (14–16). All four cardiac valves have a similar, layered architectural pattern composed of cells, including the valvular endothelial cells (VECs) at the blood-contacting surfaces and the deep valvular interstitial cells (VICs), and valvular extracellular matrix (VECM), including collagen, elastin, and amorphous extracellular matrix (ECM) [predominately glycosaminoglycans (GAGs)] (**Table 1**). Thus, the AV (and analogously the PV) has a dense collagenous layer close to the outflow surface and continuous with valvular supporting structures, which provides strength (the fibrosa); a central core of loose connective tissue (the spongiosa) rich in GAGs; and a layer rich in elastin below the inflow surface (the ventricularis).

In the closed phase of the AV, the back pressure (normally approximately 80 mm Hg) stretches the valve cusps as they come together to seal the orifice to prevent backward leakage of blood. The major stress-bearing component is collagen. Individual collagen fibers can withstand high tensile forces when taut, but collagen cannot be compressed (in contrast to the ability of elastin to stretch

Table 1 Key cellular and extracellular matrix components of the aortic valve (modified from Reference 19).

Component	Location	Putative Function	Comments, key questions
Endothelial cells	Lining inflow and outflow valve surfaces	Provide thromboresistance, mediation of inflammation	Role in transducing shear and modulating VIC function, functional differences from vascular wall EC, differences in inflow side to outflow side functions/responses largely unknown
Interstitial cells	Deep to surface, throughout all layers	Synthesize and remodel matrix elements	Currently considered the major modulator of long-term valve durability and a key mediator of disease; regional heterogeneity; regulation of activation, and the functional role of contractile potential poorly understood
Elastin	Concentrated in ventricularis layer	Extend in diastole, recoil in systole	Potential mechanistic role in disease not defined
Glycosaminoglycans (GAGs)	Concentrated in spongiosa layer	Absorb shear of relative movements and cushion shock between ventricularis and fibrosa during cyclical valve motion	Potential mechanistic role in disease not defined
Collagen	Concentrated in fibrous layer	Provides strength and stiffness to maintain coaptation during diastole	Likely the most important structural element, crimp and orientation/alignment provide directional anisotropy of properties and accommodate cyclical cuspal shape changes

and contract). Thus, although the limit to cuspal stretching and potential prolapse of the cusps into the left ventricle during diastole is the taut, aligned collagen, particularly in the fibrosa layer, the changes in shape and size of the cusps during the cardiac cycle involve changes in collagen structure beyond simple stretching and shortening. Indeed, realignment toward randomness and crimping can decrease the area of a stretched tissue layer comprised of collagen. Moreover, the directions of collagen fibers in regions of the cusps determine the directions in which the tissue has the greatest compliance (i.e., orthogonal to the collagen fiber orientation) or can withstand the greatest tensile stresses (i.e., parallel to the collagen fiber orientation). In addition, the second-to-second cyclical internal rearrangements in collagen (i.e., alignment of fibers and extension of microscopic crimp during the closed phase) are extremely sensitive to the instantaneous mechanical stresses. Rearrangements are completed early following closing (16–19).

During valve opening, the tissue of the cusps that was stretched during the closed phase (diastole) becomes relaxed owing to recoil of the elongated, taut elastin. This restores the retracted configuration of the cusp, a more random directional distribution and restored crimp of collagen fibrils, and a decreased cuspal surface area. The GAG-rich spongiosa facilitates the relative rearrangements of the collagenous and elastic layers during the cardiac cycle. Moreover, the strains during closure and the mechanical properties of the AV cusps are anisotropic (i.e., different in the radial and circumferential directions), with compliance and stretching in the radial direction greater than that in the circumferential (16).

The quantity, quality, and architecture of the VECM, particularly collagen, elastin, and GAGs, are the major determinants of not only the short-term function but also the long-term (lifetime) durability of a valve. The macroscopic mechanical stimuli that occur during normal valvular function, including both shear and solid stresses, are translated into microscopic forces that impact VIC and VEC function. These cells sense the local tissue mechanical environment (both solid and fluid) and, through valvular cell-ECM and cell-cell interactions, transduce forces into molecular changes that mediate normal valve function and pathobiology. Through such mechanisms, healthy heart valves maintain homeostasis, adapt to an altered stress state, and repair injury via connective tissue remodeling mediated by the synthesis, repair, and remodeling of several ECM components; when environmental change becomes excessive, a clinically significant valve pathology may result.

VALVULAR INTERSTITIAL CELLS

Distributed throughout all the valve layers, VICs are the most abundant cell type in the heart valves and are crucial to their function. VICs synthesize VECM and express matrix degrading enzymes [including matrix metalloproteinases (MMPs) and their inhibitors, i.e., tissue inhibitors of matrix metalloproteinases (TIMPs)] that mediate and regulate remodeling of collagen and other matrix components (16). Through these mechanisms, VICs continuously repair functional damage to collagen and the other VECM components. VICs comprise a diverse and dynamic population of resident cells. When stimulated by the surrounding mechanical environment, by certain chemical signals, or as a response to injury, VICs become highly plastic and may transition from one phenotypic state to another during valvular homeostasis, adaptation, and pathology. Although five distinct VIC phenotypes have been described, the quiescent and activated phenotypes are most important for the present discussion (16).

Adult heart valve VICs in situ have characteristics of fibroblasts; they are quiescent, without synthetic or catabolic activity for VECM. However, VIC phenotypes change with age and environmental conditions even in normal valves. For example, VICs are activated during intrauterine valvular maturation, by abrupt changes in the mechanical stress state of valves, and in disease

states, and valvular cells continuously repair a low level of injury to the VECM that occurs during physiological function (16). Cyclic stretch induces ex vivo remodeling of AV tissue (16). Moreover, either mechanical stretch (16) or transforming growth factor (TGF-β) treatment of isolated VICs (20) from mature valves induces their activation and thereby increases their synthetic activity, and the effects of stress and TGF-β on cultured aortic VICs are synergistic (20). Because the macroscopic mechanical state of the valve is likely transmitted to the VICs through their interactions with the surrounding VECM, there is considerable interest in the effects of mechanical forces on VIC function, in the mechanisms of response of VICs to their physical environment (mechanotransduction), and in the mechanical properties of isolated VICs (16, 17). We have recently demonstrated that remodeling potential of PV and AV interstitial cells are different (21). However, whether VICs in different regions of valve leaflets have different functional properties remains unknown.

VALVULAR ENDOTHELIAL CELLS

Like other regions of the circulatory system, the blood-contacting surfaces of the valves are lined by endothelial cells. The key features of VECs are summarized in Reference 16. At a basic structural and functional level, VECs resemble endothelial cells elsewhere in the circulation. However, VECs are phenotypically different from vascular endothelial cells in the adjacent aorta and elsewhere, consistent with the increasing recognition of more widespread endothelial heterogeneity across circulatory sites and the fact that VECs may interact with VICs to maintain the integrity of valve tissues and, potentially, to mediate disease (16a). For example, in response to fluid shear stress, porcine aortic VECs align perpendicular to flow, whereas endothelial cells from the nearby aorta align parallel to flow, and the transcriptional gene expression profile of aortic wall and aortic VECs are different when the different cells are exposed to the same mechanical environment. Furthermore, recent evidence indicates that different transcriptional profiles are expressed by the endothelium on the opposite (i.e., aortic and ventricular) faces of a normal adult pig aortic valve, and some investigators have hypothesized that these differences may contribute to the typical predominant localization of pathologic aortic valve calcification near the outflow surface.

Tissue Engineering Approaches to Heart Valves

Tissue engineering (TE) is an approach that attempts to combine engineering principles with the biological sciences to produce viable structures for replacement of diseased or deficient native structures. A popular method in tissue engineering is to utilize a bioabsorbable scaffolding material, seeded with autologous cells and grown in vitro, to ultimately produce a functional living tissue that can be implanted into the body. Cells form the living component produce ECM and other bioactive components [e.g., chemical signals, matrix metalloproteinases (MMPs), and inhibitors]. The ECM provides the basic framework for the tissue and is primarily responsible for the structural and mechanical properties of the tissue. The scaffold should function as a temporary cell matrix on which cells can organize, grow, proliferate, and produce native matrix.

In the case of the TEHV, the scaffold must also have a design and mechanical characteristics that allow valve-like function during in vivo maturation of the TE construct (22). The basic properties necessary for a bioabsorbable scaffolding material are nontoxicity (including degradation products), biocompatibility, resorbability, and strength (see section Scaffolds for Heart Valve Tissue Engineering). In addition, a TEHV scaffold needs the proper engineering and construction techniques to meet many of the other necessary design properties, such as geometry, density, compliance, and hemodynamics, and most of the biological activity of the TEHV should

be fulfilled by the growing cells. The polymer scaffold temporarily provides the biomechanical structural characteristics for the replacement tissue until the cells produce their own ECM that will ultimately provide the structural integrity and biomechanical profile for the replacement tissue. During this process, the scaffold will be gradually degraded, eventually leaving no foreign materials within the replaced tissues.

The Mayer group (23–31) has focused on the creation of cardiovascular structures using this tissue engineering approach. These investigators reasoned that the creation of a tissue engineered structure from autologous cells could offer several advantages over the currently available prosthetic, bioprosthetic, or homograft devices used to replace cardiac valves and arteries. These structures would be living, viable structures that could retain the normal biological mechanisms for repair and development, thereby leading to greater durability. An autologous TE heart valve could be completely biocompatible with a reduced risk of infection and thrombus formation. Of particular interest in the pediatric population, a viable TE structure could grow with the patient, eliminating the need for multiple reoperations. In addition, TE structures may prove to be less costly than currently available prosthetic devices, and there may be fewer supply limitations compared with homografts.

Working with Mayer, Hoerstrup et al. (32) implanted valve constructs into six lambs (weight 19+/−2.8 kg) (**Figure 1*a***). All animals had uneventful postoperative courses, and the valves were explanted at 1 day and at 4, 6, 8, 16, and 20 weeks. Echocardiography demonstrated mobile functioning leaflets without stenosis, thrombus, or aneurysm up to 20 weeks. Histology (16 and 20 weeks) showed uniform layered cuspal tissue with collagen and elastin and covered by endothelium (**Figure 1*b***). Environmental scanning electron microscopy revealed a confluent smooth valvular surface, with complete degradation of the polymers by 8 weeks. VECM (collagen, glycosaminoglycans, and elastin) and DNA content increased to levels of native tissue and higher at 20 weeks. This study demonstrates in vitro generation of implantable complete living heart valves based on a biomimetic flow culture system. Stock (33) also demonstrated formation of pulmonary artery tissue with histology very similar to native tissue.

More recent work by Sutherland et al. (34) utilized mesenchymal stem cells isolated from ovine bone marrow. A 50:50 PGLA/PGA blend biodegradable scaffold was utilized to create autologous semilunar heart valves and was implanted into the pulmonary position of sheep in a cardiopulmonary bypass. The valves were evaluated by echocardiography at implantation after four months in vivo, then explanted at four and eight months and examined by histology and immunohistochemistry. Valves displayed a maximum instantaneous gradient of 17.2+/−1.33 mm Hg, a mean gradient of 9.7+/−1.3 mm Hg, an effective orifice area of 1.35+/−0.17 cm^2, and trivial or mild regurgitation at implantation. Gradients changed little over four months of follow-up. Histology showed deposition of extracellular matrix and distribution of cell phenotypes in the engineered valves reminiscent of that in native pulmonary valves. As in previous PGA studies, these TEHVs functioned satisfactorily in vivo for periods of greater than four months and underwent extensive remodeling in vivo.

*Erratum

Despite this encouraging progress, we have only very limited information on the extent the TEHV duplicates the function of the native PV. For example, the gradual development of a trilayered structure, including variations in collagen, GAG, and elastin, after 15–20 weeks post implantation (35) have been reported. Yet, we do not know if these layers are functionally equivalent to the native valve and what mechanisms guide their formation in vivo. Further, we have no knowledge of the anisotropic mechanical properties of TEHV leaflet tissue and how well these compare with the native PV. In the principles of functional tissue engineering (36), it is stated that there is a need to establish the minimal functional parameters necessary to produce tissue equivalents. For heart valves, this includes quantification of the anisotropic mechanical properties of TEHV

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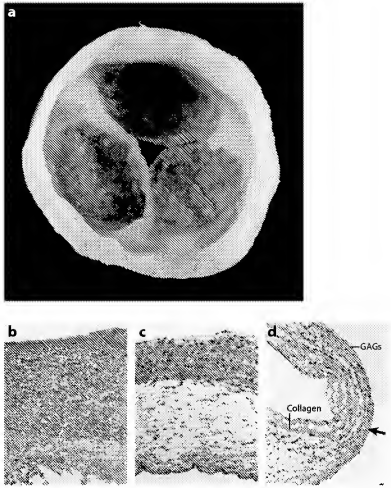

Figure 1

(*a*) An example of a trileaflet heart valve after 14 days of conditioning in a bioreactor. (*b–d*) Histology of heart valve leaflet in vivo. (*b*) At 6 weeks, there is early organization of tissue predominantly in outer layer. (*c*) Cross section of leaflet at 16 weeks shows layered cellular fibrous tissue, which is more dense near outflow surface. (*d*) Cross section of leaflet at 20 weeks demonstrates collagen (*yellow*), glycosaminoglycans (GAGs) (*blue*), and elastin (*arrow, inflow surface*). Figure reproduced with permission from Reference 28.

leaflet tissue, which can only be achieved thru multiaxial testing to determine if the developing tissue is a suitable valve replacement. Further, the degree of cellular function and similarity to the native valve has yet to be determined. Despite the fundamental nature of these requirements, we currently have no knowledge of the TEHV functional similarity, at the tissue and cellular levels, to the native PV, which will ultimately dictate functionality and long-term durability.

DESIGN CRITERIA FOR A TISSUE ENGINEERED HEART VALVE

*Erratum

In addition to meeting all the standard design criteria of a traditional tissue valve, in which biocompatibility and durability are effectively passive attributes of the materials of construction,

*This PDF amended on (3 Sept. 2009). See explanation at http://arjournals.annualreviews.org/errata/bioeng

fully laying the foundation for TEHV clinical applications requires consideration of the active behavior of the cells in the regulation of tissue growth, remodeling, and homeostasis. We must also appreciate that the mechanisms of valvular morphogenesis during embryological development are highly regulated and complex and will be difficult to stimulate in vitro. Thus, it should be readily appreciated that continued progress in this area must depend strongly on the (*a*) utilization of concepts of normal valvular and vascular development, structure, and function, including cell sorting in development, extracellular matrix biology (19), and valvular cell biology and responses to injury (16, 37, 38); (*b*) considerable understanding developed over the past several decades concerning failure modes (10, 39) and the structural basis for favorable substitute tissue valve performance (40, 41); and (*c*) basic mechanisms of physiological repair processes and the interactions of tissue valves with the host tissues (37).

Taken as whole, valve function and durability depend on the quality of collagen and other VECM constituents, VECM quality depends on VIC viability and function, VIC elaborate and remodel VECM, and VIC have a complex phenotype. Thus, the goal is to create a living tissue engineered heart valve with structural differentiation, anatomically appropriate and high quality VECM (16), viable VIC available to respond to changing physiological needs and to repair ongoing structural injury by remodeling VECM, and the capacity to grow with the patient. In the following two sections, we present an overview of two key bioengineering aspects currently of much focus in the development of TEHVs: the role of physical stimulation on TEHV tissue formation in vitro using specially designed bioreactors and the major biomechanical and biological progress in the development of biodegradable scaffolds suitable for heart valve tissues.

BIOREACTORS AND ENGINEERED HEART VALVE TISSUE FORMATION IN VITRO

Bioreactors have been developed for the dynamic mechanical stimulation of tissue engineered cardiovascular constructs, including vascular grafts (42, 43), myocardial patches (44), and heart valves (29, 45). These devices typically rely on pulsatile flow to generate a complex biomechanical environment resembling in vivo conditions and have been demonstrated to promote both the development of mechanical strength (28, 42, 43) and the modulation of cellular function (46) within these tissue engineered constructs. While these devices have shown promise in the development of functional tissue replacements (28, 42), they present several drawbacks when applied to the study of fundamental biomechanical phenomena, including small sample capacity, anatomical sample geometry, and coupled mechanical stimuli.

Few bioreactors have been designed for the explicit purpose of studying the individual effect of specific modes of mechanical stimulation on engineered cardiovascular tissues, such that candidate scaffolds and constructs can be systematically evaluated (47–50). Such devices should be designed to provide a user-defined, simple mode of mechanical stimulation, offer a sufficient sample capacity for statistically significant comparisons at multiple time points, and accommodate simple sample geometries amenable to subsequent mechanical testing. As is the case for most tissue engineering bioreactors, these devices should allow for the culture of engineered tissue samples under sterile, physiological (37°C and 5% CO_2) conditions for the duration of the experiment and should be relatively easily to clean and maintain.

In our laboratory, we are interested in studying the effects of dynamic mechanical stimulation on the development of TEHVs. Our foci are on both the evaluation of candidate scaffold materials and the conduction of mechanistic studies to elucidate fundamental biomechanical phenomena. Moreover, a long-term goal is the development of a rational basis for tissue engineering design derived from such studies, allowing tissue structure and function to be accurately predicted from

cell, scaffold, media, and environmental conditions. In separate studies, cyclic flexure, stretch, and flow (FSF) have been demonstrated to exhibit both independent and coupled stimulatory effects (47, 51–53).

As an example, even simple deformation modes can have a profound effect on forming heart valve tissues. A model system was used to determine if cyclic flexure, a major mode of heart valve deformation, has independent effects on TEHV cell and ECM development (51). In this application, ovine vascular smooth muscle cells (SMC) were seeded for 30 hours onto strips of nonwoven 50:50 polyglycolic acid (PGA) and poly-L-lactic acid (PLLA) scaffold. After four days of incubation, SMC-seeded and SMC-unseeded scaffolds were maintained either under static conditions (static group) or subjected to unidirectional cyclic three-point flexure at a physiological frequency and amplitude in a bioreactor (flex group) for three weeks. After seeding or incubation, the effective stiffness (E) was measured, with SMC-seeded scaffolds further characterized by DNA, collagen, sulfated glycosaminoglycans (S-GAG), and elastin content as well as by histology. The seeding period was over 90% efficient, with a significant accumulation of S-GAG, no significant change in E, and no collagen detected. Following three weeks of incubation, unseeded scaffolds exhibited no significant change in E in the flex or static groups. In contrast, E of SMC-seeded scaffolds increased 429% in the flex group ($p < 0.01$) and 351% in the static group ($p < 0.01$), with a trend of increased E, a 63% increase in collagen ($p < 0.05$), increased vimentin expression, and a more homogenous transmural cell distribution in the flex versus static group (**Figures 2*a*,*b***).

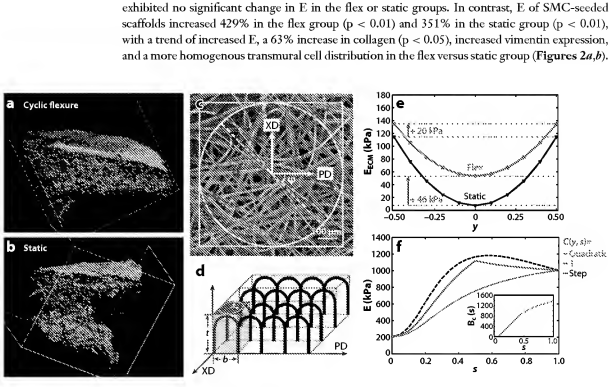

Figure 2

Three-dimensional visualization of 1 mm^3 tissue engineered heart valve (TEHV) specimens incubated for three weeks under (*a*) cyclic flexure and (*b*) static culture conditions, demonstrating that simple flexural deformations induce more robust and homogenous tissue formation. (*c*) SEM image of the planar microstructure of a 50:50 PGA/PLLA scaffold, showing the preferred and cross-preferred (XD) directions. (*d*) A schematic of the planar microstructure depicting the three-dimensional structure of the needled nonwoven scaffold in which the regions between the vertically-oriented loops of fibers that appear in panel *c*. (*e*) Transmural extracellular matrix (ECM) effective stiffness distribution predicted using a composite beam model (Equation 1), showing cyclic flexure increased the effective stiffness throughout the scaffold. (*f*) Resulting simulations of transmural ECM tissue deposition using a normalized time parameter *s*, showing that the overall TEHV effective stiffness was sensitive to the specific time-course transmural evolution of ECM. Figure adapted with permission from References 80, 110.

Moreover, a positive linear relationship ($r^2 = 0.996$) was found between the mean E and mean collagen concentration. These results show that cyclic flexure can have independent effects on TEHV cell and ECM development.

Toward quantifying the effects of mechanical stimuli similar to those that occur during normal valve function, we have developed a novel bioreactor in which FSF mechanical stimuli can be applied to engineered heart valve tissues independently or in combination (54). The FSF bioreactor consists of two identically equipped chambers, each able to hold up to 12 rectangular tissue specimens (25 × 7.5 × 1 mm) via a novel spiral-bound technique. Specimens can be subjected to changes in curvature up to 50 mm^{-1} and uniaxial tensile strains up to 75%. Steady laminar flow can be applied by a magnetically coupled paddlewheel system. Computational fluid dynamic (CFD) simulations were conducted and experimentally validated by particle image velocimetry (PIV). Tissue specimen wall shear stress profiles were predicted as a function of paddlewheel speed, culture medium viscosity, and the quasi-static state of specimen deformation (i.e., either undeformed or completely flexed). Velocity profiles predicted by two-dimensional (2D) CFD simulations of the paddlewheel mechanism compared well with PIV measurements and were used to determine boundary conditions in localized three-dimensional (3D) simulations. For undeformed specimens, predicted interspecimen variations in wall shear stress were on average ±7%, with an average wall shear stress of 1.145 dyne cm^{-2} predicted at a paddlewheel speed of 2000 rpm and standard culture conditions. In contrast, whereas the average wall shear stress predicted for specimens in the quasi-static flexed state was ~59% higher (1.821 dyne cm^{-2}), flexed specimens exhibited a broad intraspecimen wall shear stress distribution between the convex and concave sides that correlated with specimen curvature, with peak wall shear stresses of ~10 dyne cm^{-2}. This result suggests that by utilizing simple flexed geometric configurations, the present system can also be used to study the effects of spatially varying shear stresses.

Utilizing this system, we investigated the independent and coupled effects of two mechanical stimuli physiologically relevant to heart valves—cyclic flexure and laminar flow (i.e., fluid shear stress)—on TEHV tissue formation (55) using a novel FSF bioreactor (54). As previously stated, bone marrow–derived mesenchymal stem cells (BMSC) are relatively accessible and exhibit a pluripotency suitable for cardiovascular applications such as TEHVs. Recently, Sutherland et al. (34) demonstrated that BMSC-seeded TEHVs can successfully function as pulmonary valve substitutes in juvenile sheep for at least eight months. In the present study, BMSC isolated from juvenile sheep were expanded and seeded onto rectangular strips of nonwoven 50:50 blend PGA and PLLA scaffolds. Following four days static culture, BMSC-seeded scaffolds were loaded into a novel FSF bioreactor and incubated under static ($n = 12$), cyclic flexure ($n = 12$), laminar flow (average wall shear stress = 1.1505 dyne cm^{-2}; $n = 12$), and combined flex-flow ($n = 12$) conditions for one ($n = 6$) and three ($n = 6$) weeks. By three weeks, the flex-flow group exhibited dramatically accelerated tissue formation compared with all other groups, including a 75% higher collagen content of 844 ± 278 μg g^{-1} wet weight ($p < 0.05$) and an E value of 948 ± 233 kPa. Importantly, collagen and E values were not significantly different from values measured for vascular SMC-seeded scaffolds incubated under conditions of flexure alone (51), suggesting that BMSC-seeded TEHV can be optimized to yield results comparable to SMC-seeded TEHV. Moreover, we demonstrated that cyclic flexure and laminar flow can synergistically accelerate BMSC-mediated tissue formation, providing a basis for the rational design of in vitro conditioning regimens for BMSC-seeded TEHV.

Ultimately, the above mechanistic studies have to be scaled up to a functional trileaflet valve. To this end, we have developed a system to study the effects of subjecting biologically active heart valves to highly controlled pulsatile pressure and flow waveforms under sterile conditions (56). The device fits inside a standard incubator and utilizes a computer-controlled closed loop feedback

system to provide a high degree of control. The mean pressure, mean flow rate, driving frequency, and shape of the pulsatile pressure waveform can be changed automatically in order to simulate both physiologic and nonphysiologic hemodynamic conditions.

Using this device, we have investigated the effects of in vitro physiological conditioning on trileaflet tissue engineered pulmonary valves TEPVs seeded with ovine BMSC (57). A preconditioning regimen over early (three-week) and ready-for-implantation durations of six weeks were studied. The three-week duration exposed scaffold-seeded valves to static culture conditions only. At three weeks, TEPVs in the six-week groups were either maintained under static culture conditions or were transferred to the organ-level TEHV bioreactor for dynamic culturing for an additional three weeks. Hemodynamic conditions of the flow loop (using cell culture media) in the bioreactor simulated native pulmonary artery, namely a flow rate of 5 liters min^{-1} and pressure of systolic/diastolic = 35/20 mm Hg at a constant heart rate of 60 bpm. Results have indicated that physiological dynamic conditions upregulated collagen production by fourfold over the static controls. Note that the collagen content of the dynamically cultured valves was almost three times (2100 ± 478 μg/g wet weight) that of the previous FSF system study [844 ± 278 μg/g wet weight, (55)].

Collectively, these studies demonstrate the profound role of physical stimulation can have on engineered heart valve tissue formation in vitro. In addition to our approaches, others have observed and confirmed the complex role of physical stimulation on TEHV tissue formation in vitro using a variety of approaches (58–65). Clearly, these results reinforce the need for a deeper understanding of the physical parameters involved. Specifically, although it is well known that physical stimulation enhances tissue formation, the specific deformation and flow modes, temporal patterns, and relations to specific cellular signals and scaffold bulk and micro/nano architectures remain unknown. When attempting to apply these to a functioning trileaflet valve, with its complex time-varying flow and mechanical deformations, the need for the prediction and design become all the more apparent.

SCAFFOLDS FOR HEART VALVE TISSUE ENGINEERING

It is generally accepted that both chemical and mechanical factors modulate cell biosynthesis when producing ECM (20, 21, 66–68). In native tissues, multiscale modes of deformation occur that work synergistically with biochemical stimuli to determine physiologic responses. Efforts to produce viable tissue replacements that recapitulate mechanical behaviors of native tissues are confounded by the complex multi-scale architectures, the hierarchical biological phenomena, and the intricate modes of deformation typically observed in collagenous tissues. To mimic native tissue structure and organization, it is first necessary to develop techniques to produce scaffolds in a controlled manner with characteristic lengths on a scale comparable to those observed in nature.

Designing scaffolds for engineered tissues requires a bottom-up approach whereby scaffolds are produced with desired chemical, physical, and mechanical characteristics in a controlled and reproducible manner. Common physical characteristics of interest include surface texture to promote cell attachment, a highly porous microstructure to allow tissue ingrowth, and an interconnected porous network to allow adequate nutrient transport and cytokine activity, all while maintaining a desired mechanical behavior. Namely, long continuous structures with diameters on the order of native ECM (50–1000 nm) approximate the local cellular environment well. A population of fibrous structures makes them appropriate for handling tensile loads while maintaining relatively low bending rigidities. Control of the distribution of fibers during manufacturing enables the production of scaffolds exhibiting a wide array of mechanical behaviors. Furthermore, scaffolds comprised predominantly of fibrous structures provide high surface area to volume ratios and high

porosity. These characteristics encourage cell contact and the transport of nutrients as well as the removal of waste products, respectively.

Several approaches have been taken to develop scaffolds for heart valve tissue engineering, including the creation of synthetic (69) and native tissue–derived (70, 71) approaches. These are further classified into polymeric scaffolds (72), native ECM scaffolds (73–77), and collagen or fibrin gel scaffolds (60–62, 78). Although there has been good progress in all these approaches, the polymeric scaffolds have to date received the most attention and are reviewed in detail here. In particular, we focus on two major types based on the fabrication technologies used: needled nonwoven and electrospun scaffolds.

Needled Nonwovens

Polymer processing techniques originating in the textile industry have proven valuable in producing synthetic fiber meshes that are capable of stimulating isolated cells to regenerate tissue. Needled nonwovens, which are fabricated using carded polymer fibers consolidated into intertwined fiber webs with barbed needles, are highly porous and can withstand the sterilization processes necessary for in vivo use (**Figure 2c**). Isolated cells of a desired lineage can then be seeded and cultured in static or dynamic conditions. Because these highly porous scaffolds exhibit an open pore structure, the seeded cells can quickly and easily infiltrate the scaffold, producing a construct populated with cells throughout, and support tissue formation (79, 80). Flat sheets of PGA/PLLA nonwoven textile have been employed to recapitulate the geometry of the native pulmonary valve and trunk by Sutherland et al. (34).

Mechanical, needled nonwovens exhibit complex behaviors. Recently, we presented a structural model for the E of a needled nonwoven scaffold in flexure (80). The model accounted for the number and orientation of fibers within a representative volume element of the scaffold demarcated by the needling process. The spring-like E of the curved fibers was calculated using the sinusoidal fiber shapes. Structural and mechanical properties of PGA and PLLA fibers and PGA, PLLA, and 50:50 PGA/PLLA scaffolds were measured and compared with model predictions. To verify the general predictive capability, the predicted dependency of E on fiber diameter was compared with experimental measurements. Needled nonwoven scaffolds were found to exhibit distinct preferred (PD) and cross-preferred (XD) fiber directions, with an E ratio (PD/XD) of ~3:1 (**Figure 2c**). The good agreement between the predicted and experimental dependency of E on fiber diameter ($R^2 = 0.987$) suggests that the structural model can be used to design scaffolds with E values more similar to native soft tissues. A comparison with previous results for cell-seeded scaffolds (51) suggests, for the first time, that the primary mechanical effect of collagen deposition is an increase in the number of fiber-fiber bond points yielding effectively stiffer scaffold fibers. This finding indicated that the effects of tissue deposition on needled nonwoven scaffold mechanics do not follow a rule-of-mixtures behavior. These important results underscore the need for structural approaches in modeling the effects of engineered tissue formation on nonwoven scaffolds and their potential utility in scaffold design.

Electrospun-Based Approaches

Scaffolds fabricated by electrospinning natural polymers, synthetic polymers, or polymer blends have received widespread attention. Beyond the relative affordability and simplicity of electrospun natural polymers, their popularity is largely a result of a versatile manufacturing process in which slight alterations during fabrication enable the production of scaffolds with a wide array of fiber morphologies (i.e., fiber diameter, porosity, packing density, and orientation) directly influencing

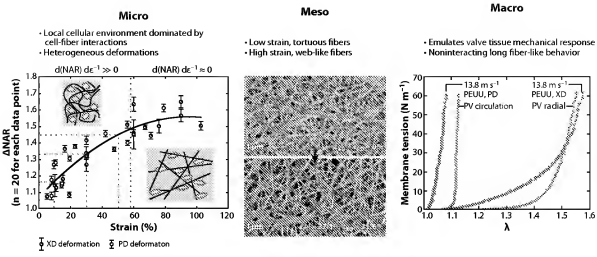

Figure 3

A schematic demonstrating how the various length scales come into play when describing ES-PEUU scaffold micro- and macromechanics. In the left panel, the nuclear aspect ratio (NAR) was used as a measure of bulk cellular deformation, which is closely coupled to local fiber microarchitecture. The figure shows a rapid increase in NAR to ~60% scaffold strain, after which a plateau was observed with further strain increases, indicating that nuclei deformations are dominated by local fiber straightening. In the central panel, the mesoscale features show the formation of web-like structures under large biaxial strains, which are consistent with the NAR results in the left panel. The right panel shows that at the tissue levels the ES-PEUU scaffolds demonstrate native pulmonary valve-like behavior.

bulk mechanical properties (81, 82). Controlled mechanical anisotropy, for example, is attained by using a rotating collection surface that induces a preferred fiber direction as the rotational speed of the collector increases (83, 84). This ability is extremely beneficial in mimicking native tissue architecture and has even been shown to approximate the highly nonlinear biaxial mechanical response of collagenous soft tissues, such as the native porcine pulmonary valve leaflet (83) (**Figure 3**).

Electrospinning produces continuous fiber scaffolds exhibiting a wide range of mechanical properties, while also providing suitable surfaces for cell proliferation and growth (85–90). A substantial amount of work can be found in recent literature concerning the mechanical and structural characterization of electrospun scaffolds (83, 89, 91, 92). Initial attempts to produce electrospun scaffolds for tissue engineering were concerned with the production and characterization of the materials, including uniaxial tensile properties and measurements of porosity and fiber diameter. Courtney et al. (83) were the first to characterize the multiaxial mechanical behavior of electrospun fabrics via planar biaxial testing. The production of a continuous fiber has the added benefit of creating multiple interrelated functional length scales, a characteristic observed in biological materials.

Electrospinning can fabricate scaffolds that possess ECM-like structures, but this morphology also results in pore sizes that are generally smaller (<5 μm) and more tortuous than those produced by other scaffold fabrication methods such as salt leaching (93) and thermally induced phase separation (94). Although it may be possible that cells seeded on the surfaces of electrospun matrices can migrate into the interior by displacing or enzymatically degrading individual fibers, an extended culture period and appropriate signals for cell migration into thick construct interiors might also be required. Thus, while electrospinning permits fabrication of biodegradable elastomeric matrices that resemble the scale, architecture, and mechanical behavior of the native ECM (95) (**Figure 3**),

achieving high cellular density and infiltration remains challenging. The Wagner laboratory at the University of Pittsburgh circumvented this problem by developing a technique to electrospin polymer fiber scaffolds while electrospraying viable cells (85).

This unique ability provided us with a unique platform to investigate cellular deformations within a 3D elastomeric fibrous scaffold (91). Scaffold specimens microintegrated with vascular smooth muscle cells were subjected to controlled biaxial stretch with 3D cellular deformations and local fiber microarchitecture simultaneously quantified. We demonstrated that the local fiber geometry followed an affine behavior, so that it could be predicted by macro scaffold deformations. However, local cellular deformations depended nonlinearly on changes in fiber microarchitecture and ceased at large strains where the scaffold fibers completely straightened (**Figure 3**). Thus, local scaffold microstructural changes induced by macrolevel applied strain dominated cellular deformations, so that monotonic increases in scaffold strain do not necessitate similar levels of cellular deformation. This result has fundamental implications when attempting to elucidate the events of de novo tissue development and remodeling in engineered tissues, which are thought to depend substantially on cellular deformations.

Translation of the scaffolds to functioning valves has begun to take shape (96, 97). Owing to the breadth of fabrication methodologies and the resulting mechanical properties, it remains unclear as to the best approach to develop a trileaflet valve. To provide some insight into the design space requirements, we conducted a basic finite element–based analysis of TEPV leaflets (**Figure 4**) under quasi-static transvalvular pressure to demonstrate the impact of mechanical anisotropy. The biaxial mechanical properties of electrospun polyester urethane urea (ES-PEUU) scaffolds were characterized and modeled using a structural constitutive model (83) that was able to accurately reproduce the scaffold mechanical properties. This model was incorporated in ABAQUS through custom written UMAT subroutines along with the material parameters of the scaffold type. Quadratic hexahedral elements were used to model the leaflet, with the leaflet geometry taken from previous models (98). The circumferential direction was taken as the preferred material direction for all simulations. Two simulations were conducted: an isotropic scaffold (wherein the scaffold fibers were randomly oriented) and a highly anisotropic scaffold (wherein the scaffold was fabricated on a mandrel spinning at 13.8 m s^{-1} to induce fiber alignment). Simulations were conducted under quasi-static loading from 0–30 mm Hg, the maximum normal functional pressure of the pulmonary valve. Finite element simulations demonstrated the major principal strain under 30 mm Hg transvalvular pressure for isotropic and anisotropic ES-PEUU scaffolds (**Figure 4**). For the isotropic case, substantial regional variations were observed. In contrast, use of a mechanically anisotropic scaffold resulted in a more uniform principal strain distribution as well as marked reduction in shear strains. Although still preliminary, these results demonstrate that modulating scaffold mechanical behavior can play a major role in determining TEPV tissue stress and potentially forming in vitro tissues. This type of stress analysis will then have to be matched to models of tissue formation to guide design-optimal physical conditioning regiments for the formation of TEHV. These are discussed in the next section.

MODELING TISSUE FORMATION

From these considerations, it is clear that critical to the application to any TEHV approach is an understanding of the time evolution of ECM stiffness and how it is modulated by physical conditioning. Although such approaches are established for a range of tissues [e.g., (99–105)], particular challenges are encountered for heart valves. In particular, valves exist in a highly dynamic, fluidic environment that induce very large tissue stresses. Moreover, these functional demands must be met at the time of implantation; no stress shielding or other protective interventions are possible.

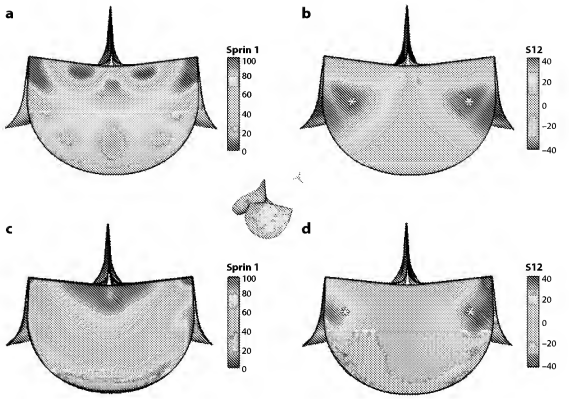

Figure 4

The effects of scaffold mechanical anisotropy on the major principal stress distributions. Using ES-PEUU scaffold materials, isotropic specimens produce highly regionally variant stress distributions with focal concentrations in (*a*) the commissure regions as well as (*b*) high shear stresses. Utilization of the 13.8 m s^{-1} anisotropic scaffold, which was the same as shown in **Figure 3** (*right panel*), produced (*c*) more uniform major principal stresses (Sprin 1 and 2) and (*d*) shear stress S12 fields, underscoring the need for both robust constitutive models and variable anisotropy for TEHV designs.

For engineered heart valve tissues, the modeling of such approaches is still in its infancy. A prudent approach is to first develop a general model for the virgin scaffold of interest and then extend it for the particular cell source utilized. As an example, we have developed a structural model for E for nonwoven scaffolds in bending (80). The model was formulated based on Freeston & Platt's "no freedom" of relative fiber motion model (106), with novel modifications introduced to describe the unique flexural mechanics of needled nonwovens. Specifically, the spring-like behavior of the crimped fibers was accounted for by calculating effective fiber stiffnesses using a model proposed by Lee & Argon (107). Structural and mechanical properties of PGA and PLLA fibers and PGA, PLLA, and 50:50 PGA/PLLA scaffolds were measured and input into the model. Details of the model are given in Reference 80. The model accounted for the number and orientation of fibers within a representative volume element of the scaffold demarcated by the needling process (**Figure 2***c*). The spring-like E of the curved fibers was calculated using the sinusoidal fiber shapes. Structural and mechanical properties of PGA and PLLA fibers and PGA, PLLA, and 50:50 PGA/PLLA scaffolds were measured and compared with model predictions. Needled nonwoven scaffolds were found to exhibit distinct preferred (PD) and cross-preferred (XD) fiber directions, with an E ratio (PD/XD) of ~3:1. The good agreement between the predicted and experimental

dependency of E on fiber diameter ($r^2 = 0.987$) suggests that the structural model can be used to design scaffolds with E values more similar to native soft tissues. A comparison with previous results (51) indicated that the primary mechanical effect of collagen deposition is an increase in the number of fiber-fiber bond points yielding effectively stiffer scaffold fibers and that the mechanics of tissue deposition on needled nonwoven scaffold mechanics do not follow a rule-of-mixtures behavior.

To simulate the formation of tissues, we continue with our focus on nonwovens owing to their well-documented ability to support tissue growth and stress-strain relations. Moreover, clinical interest in the BMSC requires a firm understanding of how these cells can be exploited to rapidly develop robust heart valve tissues. For example, cyclic flexure coupled with laminar flow can synergistically accelerate BMSC-mediated tissue formation on PGA/PLLA scaffolds, with a 75% increase in collagen concentration ($p < 0.05$) (55). It should also be noted, however, that although nonwoven scaffolds undergo permanent deformation with the application of 2–10% uniaxial tensile strain (50, 108), the relative mechanical stability of PGA/PLLA scaffolds under cyclic flexure allowed us to establish a strong positive linear relationship between effective stiffness and collagen concentration in the absence of measurable scaffold mechanical degradation (51).

Although these results suggested that E depends primarily on the gross collagen content, we also observed that cyclic flexural loading led to a more homogeneous transmural cell and ECM distribution (**Figures 2*a*,*b***). According to the elementary theory of beams (109), the outermost layers of ECM-reinforced scaffold must contribute more to the flexural rigidity of the construct than any equivalently stiff layers positioned closer to the neutral axis. Thus, it remained unclear if cyclic flexure affected only the quantity of deposited collagen or if it also affected the collagen's structural-mechanical quality (e.g., specific stiffness).

To simulate these observations, a mesoscale composite beam model that accounted for the effects of nonwoven scaffold–ECM coupling and variations in transmural collagen concentration was developed, with details (110). Briefly, the model was used to predict the ECM effective stiffness in TEHV specimens incubated under static and cyclic flexure conditions (51). Assuming elastic, locally homogeneous, isotropic material behavior, the flexural rigidity EI of a composite beam mesoscale model exhibiting a transmural effective stiffness distribution was expressed in the following integral form (110):

$$(EI)_{RVE} = \int_0^w \int_{-t/2}^{t/2} \left[R\left(C(y) \cdot B_C \cdot \tilde{E}_C\right) + E_S \right] y^2 dy dx, \qquad (1)$$

where R is an empirically-determined scaffold-ECM coupling parameter, $\tilde{E}_C$ is the effective collagen stiffness, C(y) is the normalized transmural collagen concentration distribution, and B_C is the bulk biochemically-measured collagen concentration. The parameters were each measured experimentally (51) and by imposing measured parameter values and Equation 1 as constraints, $\tilde{E}_C$ was solved for using a linear simplex method. Predicted values of $E_{ECM}(y)$ ranged from a minimum of 53.7 kPa (flex) and 7.5 kPa (static) in the center to a maximum of 134.4 kPa (flex) and 114.6 kPa (static) at the periphery (**Figure 2*e***).

This result indicated that mechanical stimulation augmented collagen intrinsic stiffness in addition to simply increasing collagen mass. This result is key in that it demonstrates that basic approaches to promote tissue formation by physical stimulation have effects beyond just the up-regulation of tissue-component mass production. The mechanical quality, which can be regulated by how the collagen is laid down (form and content), is also modified. This result is important in that it indicates means to produce more robust tissues in vitro. Moreover, these results cannot be obtained by 2D tissue systems; full 3D approaches like those used herein are clearly required to determine how cellular stimulation translates into specific ECM formation.

SUMMARY AND FUTURE RECOMMENDATIONS

To the authors' knowledge, only with some level of biological function can substantial progress be made to accommodate the unique challenges inherent in replacement valve design. It should be noted that this need is most acute in the pediatric population, for whom the options are currently very limited. Moreover, whereas the TEHV studies cited in this review have yielded insight into the events occurring during TEHV development, our efforts thus far have been largely empirical, and the mechanisms influencing the maturational process remain unknown. Challenges that face engineered heart valve researchers are significant and comprehensively involve many fields of expertise. The composite design criteria enumerated above for a TEHV are summarized in **Table 2**. All factors listed involve both basic and translational aspects and also point toward a need for focused research teams.

On a more fundamental level, any method to restore, maintain, or improve tissue or whole organ function must incorporate a thorough understanding of the intricate multiscale hierarchical arrangements typically found in nature. Engineering sustainable solutions concerned only with tissue- or organ-level function belies the multifaceted, coordinated function of these tissue structures and their constituents, which are, in turn, a result of cellular or subcellular processes that reach down to the molecular scale of protein interactions and gene transcription (16). Similarly, the development of constitutive relations for tissue function must extend beyond the macroscopic scale as well. A hierarchy of models and approaches is necessary to connect the established continuum-level relationships [i.e., phenomenological (111–115) and structural (116–123)] with underpinning cell and subcellular events. From a modeling point of view, a vital aspect of these models consists of the difficult task of seamlessly coupling various length scales.

An evolving discipline called 'functional tissue engineering' seeks to address the development of load-bearing structures in several ways (36). In particular, a critical subset of native tissue mechanical properties (14) must be selected and prioritized as design objectives (**Table 2**). This subset is important, given that the mechanical properties of the designs are not expected to completely duplicate the properties of the native tissues but to guide the remodeling process to restore function. In addition to selecting critical design criteria, it will be important to unambiguously determine engineered tissue functionality in vitro and in vivo and may require new analytical techniques. Biochemical assays are commonly used to quantify the production of new constituents but lend no insight into the structure or organization of these matrix components. Furthermore, mechanical behavior evaluations need to be placed in the context of the physiological function of the valve components and not only an idealized one-dimensional modulus, which can misrepresent the true physical behavior of an engineered tissue (14). Currently, there are numerous biocompatible materials and production techniques capable of fostering the survival and functional differentiation of a population of cells in vitro. However, we lack a sufficient understanding of the microstructural environment of a majority of these engineered materials that will ultimately dictate functionality and long-term durability. Functional tissue engineering will also benefit from advancements in the development of theoretical frameworks to model complex biological phenomena. Not only do they enable an improved understanding of intricate, hierarchically complex biological processes but they can be used to guide sound hypothesis-driven examinations of new problems and they may one day provide analytical tools to evaluate engineered implant performance in vitro and after implantation.

1. We believe that, currently, the key TEHV bioengineering challenge is to determine how biological, structural, and mechanical factors affect VECM formation and in vivo functionality. These factors are fundamental to any approach toward developing a clinically viable TEHV. Moreover, these factors must be framed in relation to the unique functional behavior of

*Erratum

*This PDF amended on (3 Sept. 2009): See explanation at http://arjournals.annualreviews.org/errata/bioeng

Table 2 Design criteria for tissue engineered heart valves

Parameter	Conventional (mechanical, bioprosthetic)	Tissue engineered
Closure of leaflets	Rapid and complete	Rapid and complete
Fluidic function	Good	Potentially identical to native valve in terms of effective orifice area and pulmonary and systemic pressure and flow levels
Risk of thrombosis	Yes (especially high in mechanical valves which require anticoagulation causing vulnerability to hemorrhage)	Endothelial surface should inhibit thrombogenesis
Surgical insertion	Easy and permanent	Easy and permanent
Risk of structural dysfunction	Degradation of materials, which are rare in mechanical valves but high in bioprosthetic valves from tissue degredation and calcification	Potentially resistant to tissue degradation and calcification owing to tissue viability
	Rare in mechanical valves	
	High in bioprosthetic valves from tissue degradation and calcification.	
Risk of infection	Ever present	Resistant
Cellular function	None	Physiological VIC and VEC function
Tissue function	Durable and stable, chemically inert, nonhemolytic	Durable and stable
		Functional anatomic characteristics: collagen, elastin, GAG distribution and structure
		Functional mechanical characteristics: anisotropy, high tensile strength, low effective flexural rigidity
		Remodel according to need
		Somatic growth
Geometry	Set by design (mechanical/pericardial bioprosthetic) or tissue source (porcine bioprosthetic)	Designed at time of implantbased patent-specific geometry
		Somatic growth adaption may require/allow for nonuniform changes
Surgical considerations	Sterility, design, and implantation procedures well established	Sterility
		Practical considerations in cell sourcing
		Implantation
		Suturing/physical handling
In vivo monitoring requirements	Standard hemodynamic function	Hemodynamic conditions over initial and long-term remodeling periods
	Degeneration/regurgitation	Cellular physiological and phenotype, mass and content levels
		Changes in tissue components and mass changes
		Structure and mechanical properties
		Differences between neonate, juvenile, and adult

heart valves and how they grow and adapt to altered physiological demands. This rationale derives from the following concepts that have resulted from work done by our group and others over the past decade: The integrity of ECM components (collagen fiber architecture, elastin, and proteoglycans) is the principal determinant of the durability of heart valves.

2. The quality of valvular ECM components depends on the constituent cellular population viability, function, and ability to adapt to different environments.

3. The long-term success of a tissue engineered (living) valve replacement will depend on its ability to function as a living valve with the capacity to maintain and remodel the ECM via mechanical signals. This is critical for scaffold design, wherein stress transfer to the ECM is largely a function of the rate of scaffold degradation in vivo.

Overall, approaches need to be structured to address these fundamental issues in order to lay the basis for TEHV that can be developed and designed according to truly sound scientific and engineering principles.

DISCLOSURE STATEMENT

ES has been a consultant to the following in the past five years: Celxcel, Direct Flow Medical, Edwards LifeSciences, Medtronic, Mitral Solutions, Pi-R-Square, Sadra Medical, St. Jude Medical, Sorin Biomedical, and Sulzer Carbomedics. There has been no discussion of the off label or investigational use of drugs or devices.

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